Photosynthesis by the Pericarp of Developing Cereal Grain

Anthony Roger Nutbeam

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Declaration

This thesis was composed by myself and describes my own original work

Contents

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Abstract

Some photosynthetic properties of the chlorophyll containing layers of the immature cereal pericarp are described.

The tissue was found to be capable of high rates of photosynthesis as determined by light dependent oxygen evolution. These rates were, however, reduced when isolated pericarps were used.

The activity and kinetic properties of the enzyme phosphoenol pyruvate carboxylase (EC 4.1.1. 31) were investigated in some detail. In general enzyme activity followed chlorophyll content of the pericarp, although these results depended upon the exact conditions of plant growth. The dependence of enzyme activity on phosphoenol pyruvate and glucose 6 - phosphate concentration was investigated. The kinetic properties of the barley pericarp enzyme were found to be very similar to that from the maize leaf.

The pericarp was found to contain high concentrations of phenols. These could be oxidised either in non-enzymic reactions by metal ions or enzymically by polyphenol oxidase. Thus extraction and reaction media were devised in which enzyme degradation by the products of phenol oxidation was minimised.

The products of carbon dioxide fixation in isolated pericarps were in part identified, and resembled those reported for plants having the C_4 pathway. Further, the measured enzyme activities correlated with those found in plants with this pathway. The metabolic events leading to the synthesis of phosphoenol pyruvate remain unresolved.

Abbreviations

ADP	Adenosine 5' - pyrophosphate
ATP	Adenosine 5' - triphosphate
CAM	Crassulacean acid metabolism
DEAE-cellulose	Diethylaminoethylcellulose
G6P	Glucose 6 - phosphate
GO	Glycollate oxidase (EC 1.1.3.1.)
HDP	Hexose diphosphate
HMP	Hexose monophosphate
K_m	The Michaelis constant
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADME	NAD specific malic enzyme (EC 1.1.1.38)
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NADPME	NADP specific malic enzyme (EC 1.1.1.40)
OAA	Oxaloacetic acid
PCK	PEP carboxykinase (EC 4.1.1.49)
PEP	Phosphoenol pyruvate
PEPC	PEP carboxylase (EC 4.1.1.31)
PGA	3 - phosphoglyceric acid
PPO	Polyphenol oxidase (EC 1.10.31)
PVP	Polyvinyl pyrrolidene
R5P	Ribose 5-phosphate
RBP	Ribulose 1, 5-bisphosphate
RBPC	RBP carboxylase (EC 4.1.1.31)
(S)	Substrate concentration
TCA	Trichloroacetic acid
v/v	unit volume per unit volume

Abbreviations (continued)

V	Reaction rate
Vmax	Maximum rate of reaction.
w/v	Unit weight per unit volume.
y	Fractional saturation of an enzyme.

Chapter 1

Introduction

The Importance of Cereals to Agriculture

In recent years, 20% of the agricultural land in the United Kingdom and 16% in the world has been devoted to the production of cereals.[50] Cereal production thus plays a major role in agricultural practice. In the United Kingdom, barley accounts for 56% of total cereal production with wheat comprising most of the remainder. These two cereals thus play an important part in the agricultural production of the United Kingdom.

Most of the world's wheat is used for human consumption as the gluten content makes it the most suitable cereal for breadmaking. Barley is used mainly as an animal feedstuff although some is malted and used in the production of various beverages, notably beer and whisky.

Due to the increasing pressure on land resources imposed by a rapidly increasing population, the amount of virgin land left to bring under agriculture is thought to be very small. Thus, the major method of increasing food production will be by producing greater yields from the same area of land. This will be achieved by improvement in cultural practice and by the development of higher yielding crop varieties.

Photosynthesis and Crop Production

In the last decade, plants have been bred which give a high yield in response to applications of fertilizer. The minerals and nitrogen which the plants derived from these fertilizers account for only 5 - 10% of the final dry matter of plants. The majority of the remainder is derived from photosynthetic carbon dioxide fixation. Zelitch[131] suggested that breeding programmes designed to increase the efficiency of photosynthesis of crop plants will produce high yielding varieties.

In his discussion of the improvements which may result from such a programme he confines his attention solely to leaf photosynthesis. However, as will be described later, there is much evidence that the leaves of cereal plants have only a minor role in the production of dry matter for deposition in the grain.

Grain Filling

Dehérain and Dupont[38] observed that during the period of grain starch deposition many of the lower leaves of the cereal plant were senescent. This led to the theory that starch was stored in the leaves prior to ear emergence and that after this the starch was transferred to the developing grain.

Archbold and Mukerjee[5] showed that the dry weights of the leaves and leaf sheaths remained constant during grain filling, but that the loss of dry weight from the stem was much lower than the increase in the dry weight of the ear. However, the total dry weight of the plant increases 20 - 30% after ear emergence[144]. It was therefore concluded that photosynthesis occurring after ear emergence is responsible for grain filling, and not transfer of starch from elsewhere in the plant.

Methods of Determining the Relative Contribution of Parts of the Plant to Grain Filling

I Shading and Defoliation

Many attempts have been made to assess the relative contribution of photosynthesis in the leaves, stems and ears of cereal plants to the dry matter of the grain. Initially this was done by shading the organ and comparing the yield against that of a control plant with the organ unshaded. For varieties of barley, the relative contribution of the ears was 19 - 28% (Spratt Archer[144]), 30% (Plumage Archer[4]), and 2 - 33% (Proctor[133]).

In wheat the ear contribution was 26% (Peko[117]), 25% (Jufy[117]),

10 - 44% (Sabre[85]), and 18 - 46% for six varieties of Indian wheat[7].

In general, the weight of grain produced by a shaded ear was 30% below that of an unshaded ear. Under these conditions it was assumed that the only effect of shading was to prevent photosynthesis by the ear, thus this figure should represent the contribution by photosynthesis of the ear to grain filling. However it was noticed by Watson and Norman[144] that the time taken for the grain of shaded ears to reach maturity was less than that of unshaded ears. Thus, shading may reduce the time available for deposition of dry matter in the grain. This may result in an over estimation of the contribution of the ear to grain filling.

Most of the photosynthesis which results in grain filling other than that of the ear itself, takes place in the flag leaf (i.e. the leaf immediately below the ear). The lower leaves appeared to play little part in grain filling[4], in fact their removal has been shown to increase yield. In one experiment Archbold[4] estimated the relative contribution of the leaves by compairing the yield of control plants with that of plants with leaves removed. She noticed that plants with reduced leaf number produced fewer tillers (ear bearing stems). Since yield is calculated as weight of grain x no. of grain per ear x no. of ears per plant, the yield was reduced, in this experiment, both by a reduction in grain weight and a reduction in the number of ears per plant. Thus the contribution of the leaves to grainfilling was over estimated. The shading of leaves may also have led to a reduction in the number of tillers. There are therefore some serious limitations to the technique of ear shading in the estimation of the contribution of ear photosynthesis to grainfilling. Buttrose and May[27] express doubts as to the validity of these results. They suggest that when

photosynthesis in a particular organ is decreased, photosynthesis in other organs may increase. The reduction in yield may not, therefore, relate directly to the inhibition of photosynthesis in a particular organ. However, shading does measure the effect of light deprivation on the weight of grain produced, which after all is the most important economic factor in grain production.

II Carbon Dioxide Fixation

(i) 14 - Carbon Dioxide ($C^{14}O_2$)

Quilan and Sagar[116] fed 14-carbon dioxide ($C^{14}O_2$) in turn to leaves and ears of wheat. The presence of radioactivity was detected by autoradiography. 14 - Carbon (C^{14}) fixed by the lower leaves was mostly retained within them. Although a small proportion was transported down towards the roots, no radioactivity was detected in the ear. When $C^{14}O_2$ was fed to the ear and flagleaf, it was retained by these organs, except just after anthesis, when presumably the ability of the grain to accept photosynthate was not very great. Birecka and Skupinska[18] showed that shading the ear caused a reduction in $C^{14}O_2$ fixed by whole plants of barley and concluded that the ear was responsible for 30% of the photosynthesis of the plant. From the above it can be seen that $C^{14}O_2$ fixation is decreased by 30% as was the yield in previous experiments. Thus yield reduction by shading could principally be due to inhibition of photosynthesis.

Buttrose and May[27] found that $C^{14}O_2$ fed to barley ears was detectable in grain at harvest. Label was retained in the pericarp when feeding took place 3 - 9 days after anthesis, but at later stages it was found in the endosperm. The presence of starch in the pericarp at the early stages of development has been confirmed by MacGregor et al.[97] This indicates the importance of the pericarp as a store for carbohydrate

in the early stages of grain development.

(ii) 12 - Carbon Dioxide

The weight of CO_2 recovered from a stream of air passed through a chamber containing an ear of Spratt Archer barley was compared with that recovered when the chamber was empty[115]. The amount of carbohydrate which could have been derived from theweight of CO_2 fixed by the ear was estimated. This was equivalent to 34% of the dry weight of the grain.

Thorne[133], in a similar experiment measured the concentration of CO_2 in the effluent gas with an infra-red gas analyser and both CO_2 uptake and CO_2 production by the ear were recorded. She found that respiratory losses accounted for a large proportion of the CO_2 fixed. In barley, CO_2 equivalent to 80% of the grain's final dry weight was fixed by the ear , and CO_2 equivalent to 50% of the final grain weight was fixed by the flag-leaf. The excess of 30% fixed was concluded to be the amount lost by respiration.

In the case of wheat, CO_2 equivalent to 17 - 30% of the final grain weight was fixed by the ear[133], which was equal to the amount lost by respiration. It would be incorrect, however, to conclude that the net contribution of the wheat ear was zero. If the contribution of the ear was prevented (e.g. by shading) respiratory loss would account for a significant proportion of the photosynthate transferred to the grain from the flag leaf. Thus the contribution of the ear may still be significant.

Recent estimates of the contribution of the wheat ear have been as high as 66%[129]. The discrepancy between this and Thorne's estimations may have been due to varietal or environmental differences.

III Intergrain Competition

An ingenious method of estimating the contribution of the ear to grain filling, which was thought to give a definitive result, was described by Buttrose and May[27]. In preliminary experiments, either the upper or lower half of the immature ear was supplied with $C^{14}O_2$. No radioactivity was found in the half not receiving $C^{14}O_2$, thus it was concluded that carbon, once present in the grain, is not transported elsewhere. Non-grain assimilate i.e. photosynthate from the leaves, stem and tissues of the ear other than the grain (non grain parts of the plant) (Fig. 1.1) was ass umed to be supplied at a constant rate to the ear, irrespective of the number of grains in the ear.

A control plant (experiment 1) was compared with one where half the grains (including paleae and lemmas)(see Fig. 1.2) were removed from the ear (experiment 2). The contribution of the non-grain assimilate to each of the remaining grains was assumed to double.

Thus, if n_1 = number of grains in experiment 1

n_2 = number of grains in experiment 2

w_1 = average weight of grains (g) in experiment 1

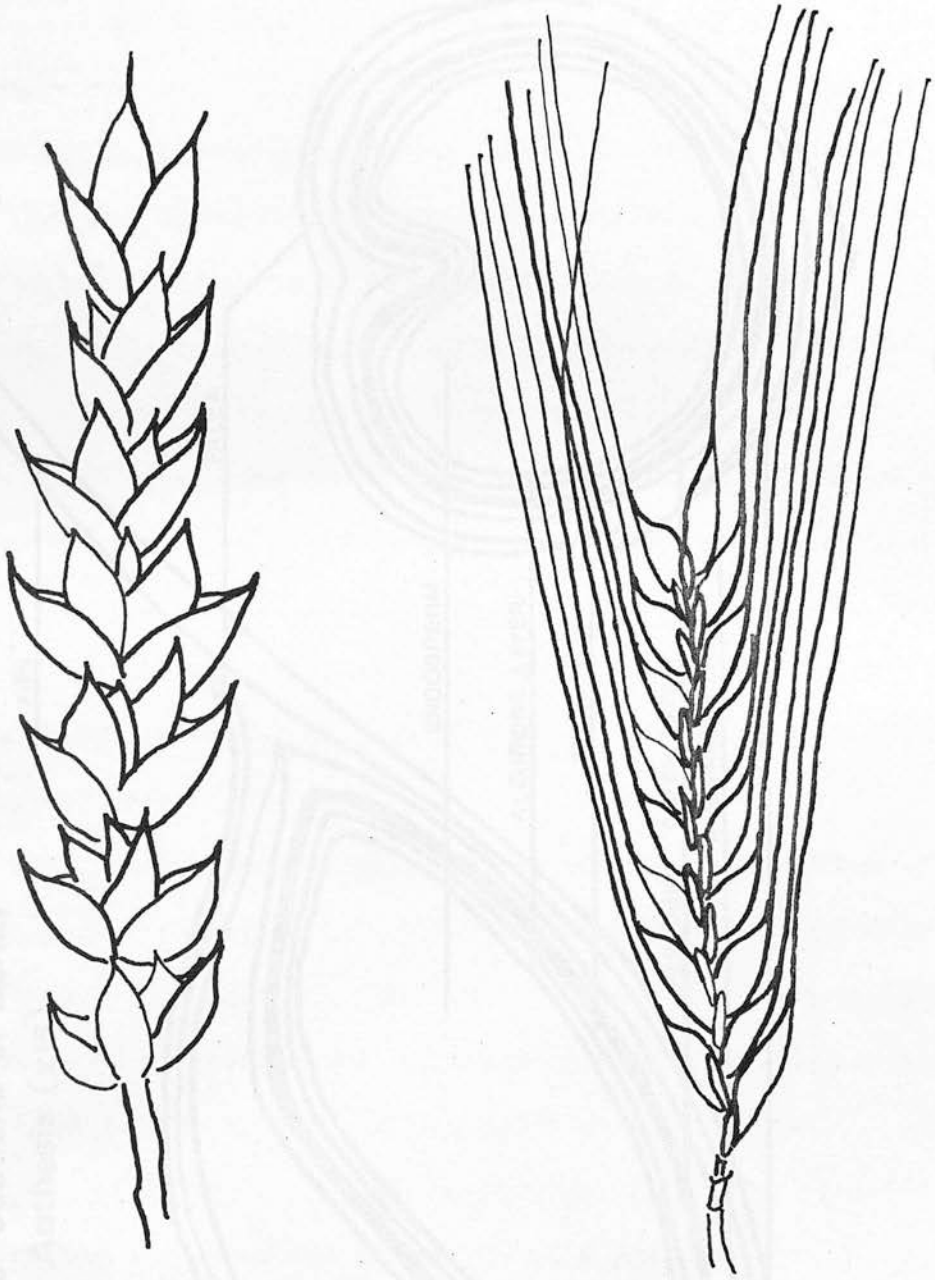
w_2 = average weight of grains (g) in experiment 2

x = the average contribution (g) of a grain to its owm weight

then - $$n_1(w_1 - x) = n_2(w_2 - x) \quad .$$

Using this relationship the contribution of the grain of barley (var. Prior) was estimated to be 64% in one experiment and 76% in another[56]. This work indicates a much higher contribution by the grain than in those methods discussed above. This could be explained if the parts of the ear other than the grain made no contribution at all. Indeed they may be responsible for the respiratory losses discovered by

Figure 1.1 Ears of Wheat and Barley

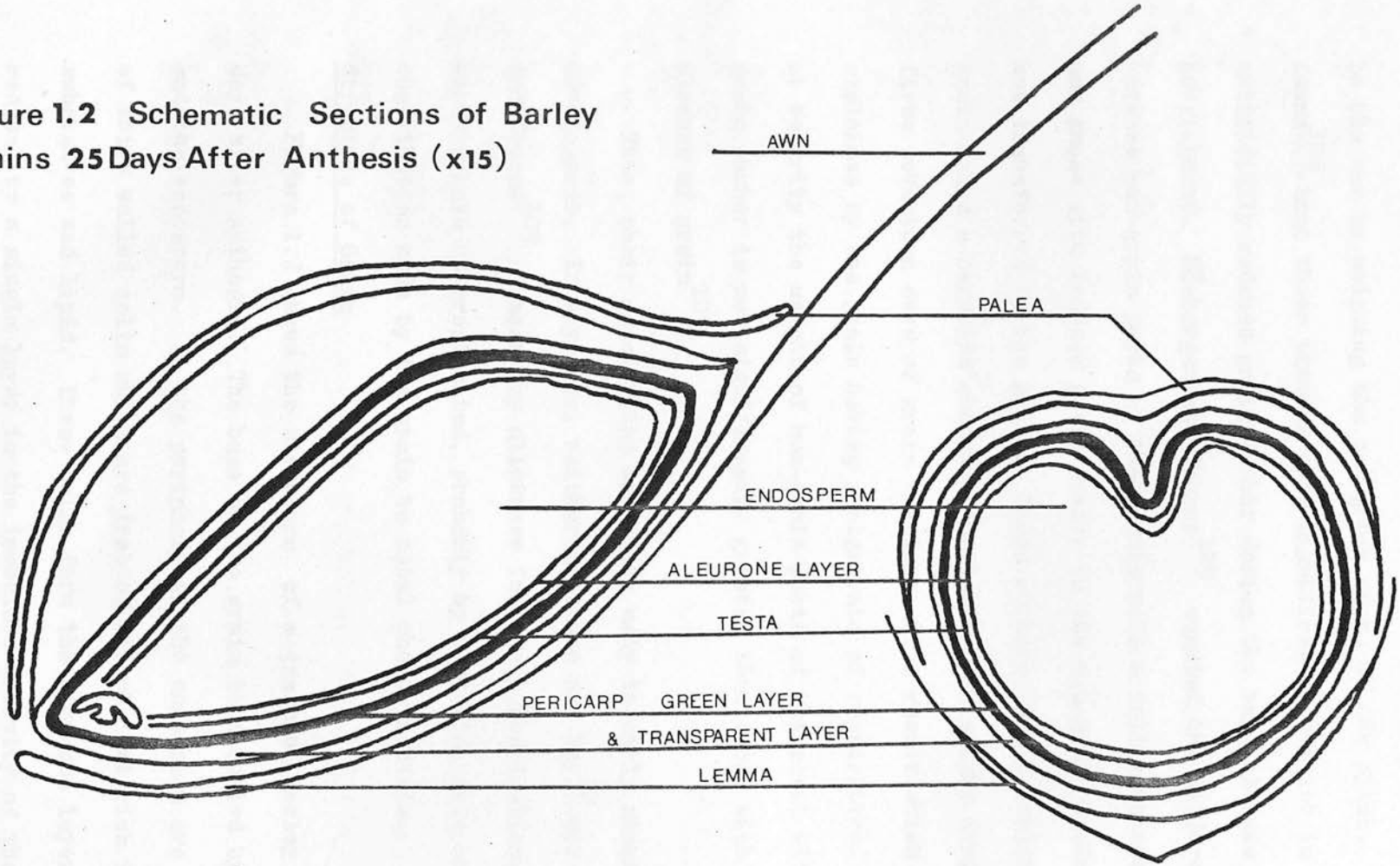


Wheat

Barley

(x1·5)

Figure 1.2 Schematic Sections of Barley Grains 25 Days After Anthesis (x15)



Longitudinal

Transverse

Thorne[133].

Nösberger and Thorne[109], tested the assumption of Buttrose and May[27] that removal of the grain did not alter the rate of supply of dry matter to the ear by weighing the non grain parts of the plant. It was found[109] that these tissues were significantly heavier in plants with artificially reduced grain number during the early stages of grain development. Nösberger and Thorne[109] equated the difference in weight between non-grain parts of the plants with a full complement of grain and those with reduced grain number to the non-grain assimilate which is not transferred to the grain. These authors then calculate that the grain makes a negative contribution to its own weight during the first seventeen days of grain development, a result which could be explained by the grain having a high rate of respiration. However, at maturity the weight of non-grain parts of the plant with reduced grain number is not significantly greater than those with a full complement of grain[109].

Thus, their conclusions may apply only to early stages of grain development. In any case, neither Buttrose and May[27] nor Nösberger and Thorne[109] made any allowance for grain respiration. Thus, these workers have underestimated, probably by a considerable amount, the contribution made by the grain to total photosynthesis.

Structure of Grain

Figure 1.2 shows the structure of a grain of barley twenty-five days after anthesis. The base of the grain is occupied by the embryo[17], and the endosperm. At the periphery of the endosperm are several layers of thick walled cells which are free of starch, but rich in nitrogenous substances and lipid. These cells form the aleurone layer, and are reduced to a single layer in the immediate vicinity of the embryo[17] and in the furrow of the grain[55]. In contrast the aleurone layer

is only one cell thick in wheat[111].

Outside the aleurone layer and in close contact with it is the testa. This is enclosed by the green layer of the pericarp and this in turn is surrounded by the colourless layer of the pericarp. Enclosing the whole are the palea and lemma (which may be awned). These form the husk of the mature grain and may or may not adhere to the outer layer of the pericarp. The chlorophyll containing tissues of the grain are the palea, lemma and the green layers of the pericarp.

The Palea and Lemma

I The Awn

It was noticed by many workers that the contribution of the ear was greatest in awned varieties of cereals[27,49,133]. However, Asana and Mani[7] studied a selection of Indian wheats and found no correlation between the presence of awns and increased contribution to grainfilling. Vervelde[139] found that removing the awns from wheat, rye and barley decreased the yield by 4 - 6%, 1 - 2% and 2 - 5% respectively. An extensive study of Lamb[91] showed that awned varieties of wheats produced grains on average 1 - 4% heavier than non awned varieties. The awns may make only a minor contribution to production of grain dry matter. It is possible that this contribution is not photosynthetic since it has been suggested by Miroslavov[104] that the major function of the awn may be to increase transpiration. This would promote the flow of nutrients from the soil (assuming adequate soil moisture content) and ensure a supply to the ear. In conditions of drought however the presence of awns may decrease yield. Certainly Teare et al.[129] found that the ratio of photosynthesis to transpiration was 20% greater in awnless varieties of wheat. Schaller et al.[119] in a study of four isogenic lines of Atlas barley, differing only in awn size,

showed that the presence of full awns contributed to yield, especially in high yielding soils and climates, however, in areas which produced low yields the half-awned varieties produced the greatest yields. McKenzie[98] has shown recently that the presence of awns in two varieties of wheat actually decreased yield. Thus, there is much diversity of opinion on the function and contribution of the awn.

II The Immature Husk

It was observed by Miskin and Rasmusson[105] that the lemmas and paleae of barley had few stomata. This led these authors to conclude the the grain was unimportant in terms of CO_2 fixation. They assumed that the only source of CO_2 was the atmosphere. However, it is possible that the grain refixes CO_2 produced by respiration. If this were the case, few stomata would be advantageous, preventing the escape of CO_2.

There are no stomata on the inner surfaces of the paleae and lemmas of wheat[128]. The inner surface of the husk may therefore provide resistance to the outward diffusion of CO_2.

The Pericarp

If the CO_2 fixed by the grain is derived from respiration within the grain itself, the green layers of the pericarp are ideally situated to trap it. This tissue is in close association with the endosperm which, during the middle and later stages of development, consists of the bulk of the grain (see Fig. 1.2) and is presumably responsible for the greater part of grain respiration.

The green layer of the wheat pericarp has been shown to contain the vascular tissue which supplies the developing endosperm and embryo with nutrient[55]. This vascular tissue runs along the furrow of the grain in close association with the endosperm as the aleurone layer in

this region is not continuous (Fig. 1.3). This may provide a simple route for pericarp assimilate to return to the endosperm. The vascular tissue does not appear to be enclosed by a bundle sheath, as is the case in C_4 plants[39].

The green layer of the pericarp contains chloroplasts[145]. Twenty five days after anthesis many of these well developed chloroplasts are found to contain starch[145]. Isolated barley pericarps are capable of light dependent oxygen evolution, using dichlorophenol indophenol as an electron acceptor[13], indicating that the chloroplasts in this tissue are active. Furthermore, Evans and Rawson[49] have shown that intact grains with the husk removed are capable of light dependant carbon dioxide fixation. Thus it appears that the green layer of the pericarp is capable of photosynthesis.

Development of the Grain

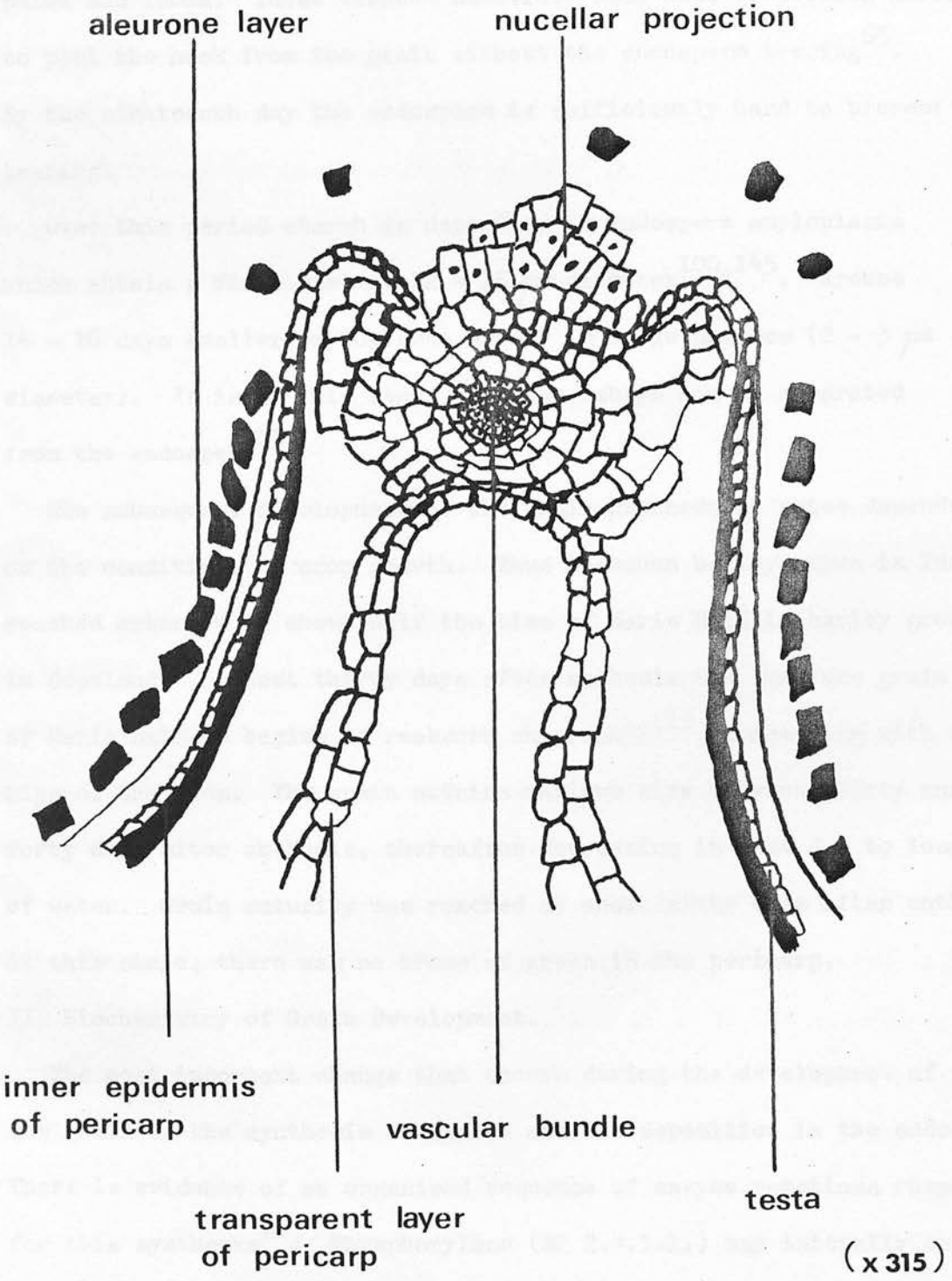
From the time of fertilization of the ovule the grain grows rapidly. Its development is characterised by a series of changes in morphology and metabolism.

I Morphological Development.

During the first five days following fertilization the growth of the outer layers i.e. pericarp and testa accounts for most of the growth of the grain[65]. The endosperm during this stage develops first into a mass of free nuclei. Subsequently a layer of nuclei on the periphery develop into the aleurone layer. Cell walls are formed between the nuclei, around 1-2 days after anthesis, such that the endosperm develops normal cellular structure[132]. Mitochondria can be seen in these cells throughout the early and middle stages of development.

The rate of pericarp growth decreases about the seventh day and at this stage the grain becomes bright green in colour. This is due to

Figure 1.3 Magnified Transverse Section of the Vascular Region of a Wheat Grain[55]



the green layer of the pericarp showing through the outer transparent layer and the translucent husk. From about this time the endosperm begins to store starch and will therefore stain blue with iodine[13].

About the tenth day the grain has developed to the extent that the transparent layer of the pericarp touches the inner surfaces of the palea and lemma. These tissues associate such that it becomes difficult to peel the husk from the grain without the endosperm tearing[65]. By the eighteenth day the endosperm is sufficiently hard to prevent tearing.

Over this period starch is deposited in endosperm amyloplasts which attain a final size of 12 - 15 μm diameter[100,145]. Around 14 - 16 days smaller amyloplasts appear in large numbers (2 - 3 μm diameter). It is at this stage that the embryo can be separated from the endosperm[13].

The subsequent development of the grain proceeds at rates dependent on the conditions of crop growth. Thus Hannchen barley grown in Idaho[65] reached maturity in about half the time of Maris Baldric barley grown in Scotland[13]. About thirty days after anthesis the immature grain of Maris Baldric begins to reabsorb chlorophyll[13], commencing with the tips of the awns. The grain attains maximum size between thirty and forty days after anthesis, thereafter decreasing in size due to loss of water. Grain maturity was reached at about sixty days after anthesis. At this stage, there was no trace of green in the pericarp.

II Biochemistry of Grain Development.

The most important change that occurs during the development of the grain is the synthesis of starch and its deposition in the endosperm. There is evidence of an organised sequence of enzyme reactions responsible for this synthesis[14]. Phosphorylase (EC 2.4.1.1.) may initially catalyse the formation of the short chains of glucose molecules which is one

of the substrates for the enzyme starch synthetase (EC 2.4.1.11). This catalyses the sequential transfer of glucose units from nucleotide sugars to the preformed chains.

That the endosperm is the site of respiratory metabolism (observed in whole grain by Thorne[133], Evans and Rawson[49]) is indicated in barley by the presence of a number of enzymes of the Embden-Meyerhof-Parnas and pentose phosphate pathways[42]. The activities of these enzymes are initially low but rise with time reaching a maximum at approximately half way through the development of grain. The enzymes of the pentose phosphate pathway maintain quite high activity in the later stages of development, but those of the Embden-Meyerhof-Parnas pathway fall to low levels of maturity.

A peak in respiratory activity halfway through the development of grain has also been reported for wheat[49]. It is interesting to note that Evans and Rawson[49] found that in wheat throughout grain development photosynthesis by the pericarp almost equalled the rate of respiration of the grain (with husk removed). In barley the chlorophyll content of the pericarp follows a similar time course to that of the activity of the enzymes of the Embden-Meyerhof-Parnas pathway. Thus, in both wheat and barley, the rate of endosperm respiration and pericarp photosynthesis may be linked.

The activities of a number of pericarp enzymes have been estimated during development[1, 97]. Phosphorylase and α- amylase activity rise rapidly following anthesis reaching a maximum at about the twelfth day, thereafter falling to very low levels. At each stage of grain development the pericarp α- amylase activity is greater than that of the endosperm[1, 97], while pericarp phosphorylase activity is greater than that of the endosperm only in the first fifteen days following anthesis. The significance of these findings is still not clear. MacGregor et al[97]

suggested that the pericarp may function as a temporary starch store in the early stages of grain development. This is an acceptable theory since during the first few days following anthesis the pericarp increases in size at a much greater rate than the endosperm. When the rate of growth of the endosperm is such that it could synthesise a greater quantity of starch, precursors may be transferred from the pericarp. At present there is little evidence for this transfer.

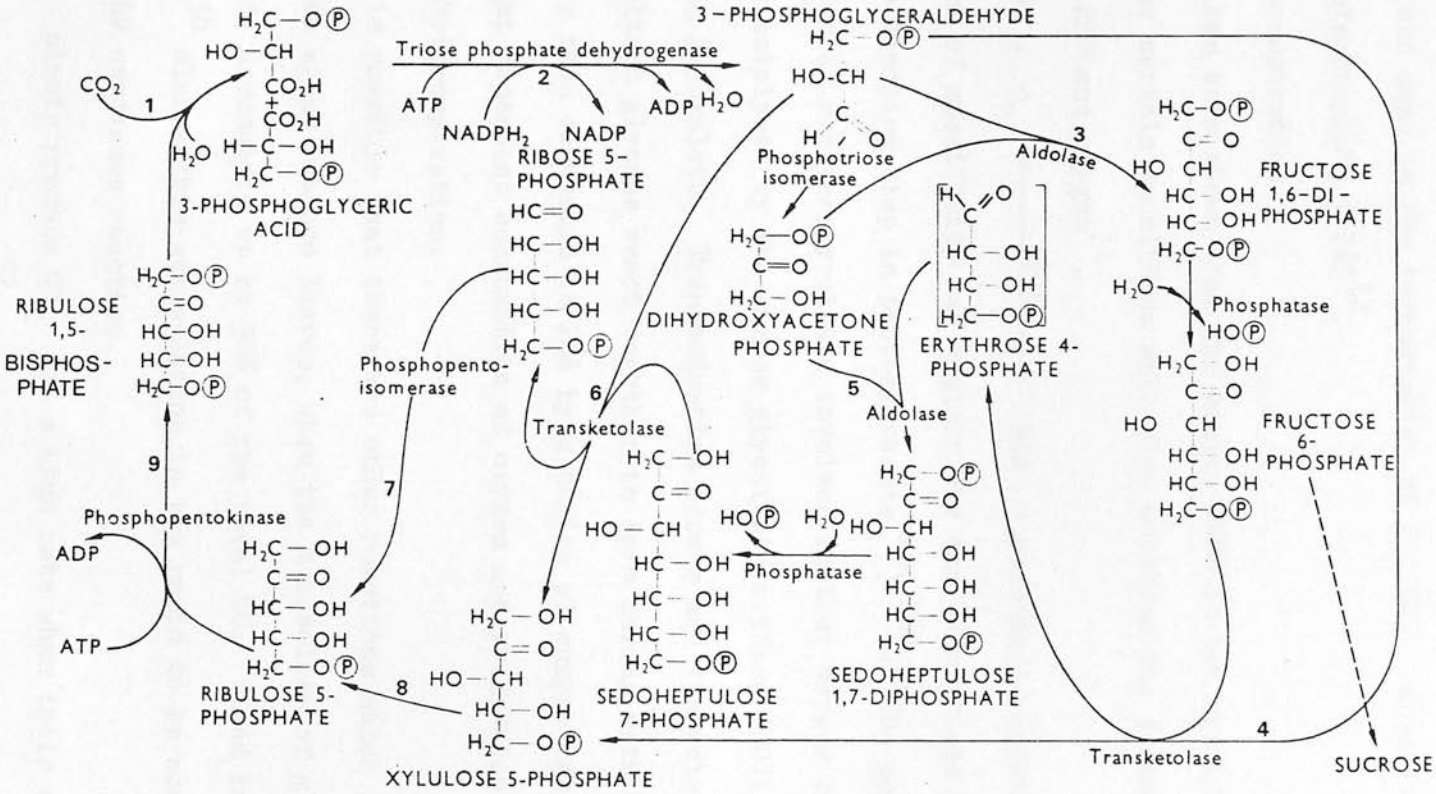
Carbon Dioxide Metabolism

It is obvious that complicated control processes are involved in the sequence of events which occur between anthesis and grain maturity. This present work is concerned with elucidating the photosynthetic function, if any, of the cereal pericarp. The pericarp is also an important translocating tissue and may possibly also have a function as a store for starch in the initial stages of grain development. These functions combined with the unique environment and morphology of the pericarp may result in this tissue having some unusual photosynthetic properties.

Most of the previous research on photosynthesis has been carried out on plant leaves, and other tissues have received little attention. Although present knowledge may not be applicable to the pericarp, photosynthetic properties may be similar to that of the leaf, so a brief review of leaf photosynthetic CO_2 fixation will now follow.

I The Calvin cycle (Fig. 1.4)

This has been found in the leaves of all plants so far examined[140]. CO_2 is fixed by the enzyme ribulose 1, 5 -bisphosphate carboxylase(RBPC) (EC 4.1.1.39). In plants which have only the Calvin cycle of CO_2 fixation, the first detectable product of photosynthesis is 3 - phosphoglyceric acid (PGA) a C_3 acid. Such plants called C_3 plants, include most of those adapted to growth in a temperate climate e.g. pea, barley,



Photosynthetic carbon reduction cycle (Calvin cycle). ↑, Drive from light reaction.

Figure1.4

wheat, rice, tobacco, and sunflower. The PGA is subsequently reduced to 3 - phosphoglyceraldehyde, two molecules of which condense to form hexose diphosphate (HDP). Some of the HDP is used in synthesis of sucrose and some in the regeneration of the CO_2 acceptor ribulose 1, 5 - bisphosphate (RBP)[12].

II Photorespiration

It has been shown that the enzyme RBPC is not specific for CO_2 and under certain conditions will also catalyse the following reaction between RBP and oxygen[24].

$$RBP + O_2 \xleftarrow{RBPC} PGA + \text{phosphoglycollate}$$

This lack of specificity may explain why the carboxylase reaction was considered the rate limiting step in photosynthesis [19, 141]. The subsequent metabolism of phosphoglycollate involves another oxygen consuming reaction catalysed by the enzyme glycollate oxidase (GO)(EC 1.1.3.1.) producing glyoxalate. Transamination occurs and two molecules of the resultant glycine react together to form serine with CO_2 evolution. Serine is then converted to PGA by a series of enzyme catalysed reactions[137]. This light dependent consumption of oxygen and production of CO_2 is called photorespiration.

It is possible that there are other reactions which give rise to glycollate since tobacco leaves, when the metabolism of glycollate is inhibited, accumulate up to 50% of the total CO_2 fixed into glycollate. Zelitch[150] claims this accumulation is too rapid to be accounted for by the RBP oxygenase reaction.

C_3 plants produce CO_2 at a high rate when their supply of light is suddenly extinguished[37]. This phenomenon is called the post illumination burst. After a few minutes the rate of CO_2 production decreases to that of dark respiration. Thus, photorespiration does not appear to be 'turned off' by darkness as quickly as photosynthesis.

III Crassulacean Acid Metabolism (CAM)

A group of succulent plants, the Crassulaceae well suited to the arid environment of their natural habitat have a unique system of CO_2 fixation. During the day, the stomata of these plants are closed which prevents not only excessive water loss by transpiration but also the entry of CO_2 for photosynthesis[107]. At night, when the temperature is such that transpiration is low the stomata open. The enzyme phosphoenol pyruvate carboxylase (PEPC)(EC 4.1.1.31)[126] then catalyses the following reaction:

Phosphoenol pyruvate (PEP) + CO_2 + H_2O $\xleftarrow{\text{PEPC}}$ Oxaloacetate (OAA) + H_3PO_4

The OAA is then reduced to form malate. Thus $C^{14}O_2$ which is fixed in the dark is found principally in malate. On placing the plant in the light for two hours much of the $C^{14}O_2$ is found in sucrose[88]. The transfer of label from malate to sucrose appears to occur by decarboxylation of malate (e.g. by malic enzyme (EC 1.1.1.40) followed by Calvin cycle fixation of the CO_2. Few plants of agricultural importance have this pathway, the one exception being the pineapple[107].

IV C_4 Metabolism

(i) C_4 pathway

This group ofplants also has an auxillary carbon dioxide trapping mechanism catalysed by PEPC. Unlike the Crassulaceae significant CO_2 fixation by PEPC takes place in the light. OAA is the first product of photosynthesis that can be detected[69]. This is rapidly reduced to malate or aminated to aspartate. These C_4 acids are thus thought to be transported to the site of the Calvin cycle. Here CO_2 is released and then refixed by RBPC. As the C_4 acids are the first detectable products of CO_2 fixation, plants with this type of pathway are called C_4 plants. They include high yielding tropical grasses, such as sugar cane,

maize and sorghum, and many weeds adapted to tropical climates.

(ii) Anatomy.

Leaves of C_4 plants differ from leaves of C_3 plants in their specialised anatomy[39]. The vascular tissue of the leaves is enclosed by a layer of cells called the bundle sheath which is thought to be the site of secondary fixation of CO_2 by the Calvin cycle[39].

(iii) Photorespiration in C_4 plants.

The post illumination burst observed in C_3 plants has not been observed in maize (C_4) leaves[138]. This may indicate that C_4 plants lack photorespiration. However, while CO_2 may not be evolved in the light from C_4 plants, it may still be produced by pathways of photorespiration, only to be refixed before release by PEPC. Certainly, there is some evidence that C_4 plants are capable of photorespiration as the enzymes which catalyse photorespiration have been found in C_4 plants but at much lower activities than in C_3 or CAM plants[136].

(iv) Carbon Dioxide Compensation Point.

When tissues capable of photosynthesis are put in a closed system at constant temperature, and in the light, the CO_2 concentration of the air decreases to a constant level called the compensation point. At this steady state the rate of photosynthesis is equal to the rate of CO_2 production. C_3 plants are able to reduce the concentration to $5 \times 10^{-3}\%$. C_4 plants reduce the CO_2 concentration further, by a factor of 10 to $5 \times 10^{-4}\%$[40]. This provides further evidence that the rate of photorespiration in C_3 plants is greater than that of C_4 plants.

(v) Air Space and Rate of Translocation.

The air space between cells of C_4 leaf appears to be much smaller than in C_3 plants[28]. This is likely to be a manifestation of the specialised anatomy. A survey of C_4 and C_3 plants showed that leaves of sorghum and millet (C_4 plants) had higher rates of translocation

than soybean, tobacco, radish, and tomato leaves (C_3)[74]. High rates of translocation have also been reported in the leaves of maize[44] and sugar cane[66]. These may be due to the increased vascular tissue in C_4 plants observed by Crookston and Moss[36]. Alternatively, because of greater cell density, the increased number of vascular bundles may be necessary to ensure an adequate supply of nutrient from the roots.

(vi) The Rate of Photosynthesis in C_4 plants.

It appears that a complete co-ordination of anatomical and biochemical properties of the leaf is required to obtain the high rates associated with C_4 photosynthesis[47]. In studies with hybrids of C_4 and C_3 species of Atriplex[21], the high rates of photosynthesis could not be explained solely in terms of specialised anantomy since one hybrid with specialised anatomy but lacking high levels of PEPC did not fix CO_2 at high rates. In fact, not one hybrid (each hybrid had a range of C_4 and C_3 characteristics), had completely functional C_4 photosynthesis.

In C_4 photosynthesis, fixation by PEPC is thought to take place in the cells that surround the bundle sheath, that is the mesophyll[87]. The Calvin Cycle enzymes have been found by Slack and others[122] to be present only in the chloroplasts of bundle sheath cells. The enzymes of C_4 acid decarboxylation are also thought to be located principally in the bundle sheath cells[45,87]. These findings suggest that the C_4 acids migrate to the bundle sheath from the mesophyll where they are decarboxylated and the CO_2 fixed by the Calvin cycle.

On the other hand, Coombs[31] has suggested that the Calvin cycle enzymes are located in mesophyll chloroplasts and the bundle sheath cells store starch. Certainly, enzymes of starch and sucrose synthesis have been found in both mesophyll and bundle sheath cells in C_4 grasses[25]. Evidence for this scheme comes from the work of Laetsh and Kortschak[90] who were able to show starch and sugar synthesis in cultures of the C_4

plant Froelichia gracilis. The culture was initiated from the stem of the plant and had been cultured for 18 months prior to the experiment. Thus, despite the similarity of the chloroplasts in the callus to mesophyll chloroplasts, it is not possible to confirm that the callus and mesophyll cells were identical. Since the callus was homogenous in cell type it would appear that the coexistence of mesophyll and bundle sheath cells are not necessary for the synthesis of sucrose and starch. The cultured cells may have had properties of both the mesophyll and bundle sheath which is possibly an artefact of the technique. Thus while this would support the simpler scheme described by Coombs[31] more evidence is required before it can be endorsed. If the Calvin cycle occurs in the bundle sheath only, the transport of C_4 acids to this tissue must be a rapid process in order to keep pace with the high rate of photosynthesis. If the mesophyll cells synthesise sucrose, it is difficult to ascribe the bundle sheath a function although it may act as a reservoir for assimilate formed in excess of the capacity of the vascular bundle to carry it away.

Both schemes require the presence of PEPC in the mesophyll which is indeed found at high activity in this tissue[121]. It is possible that this enzyme reduces the concentration of CO_2 in the mesophyll cells to very low values. Thus, the CO_2 concentration gradient between the tightly packed cells and the stomata (at which the concentration would approximate to atmospheric) would be greater in C_4 than C_3 plants. This high concentration gradient which would increase the inward flow of CO_2 may explain the high rates of photosynthesis observed in C_4 plants.

(vii) High temperature and Light Intensity Optima.

Most C_4 plants have their temperature optimum for photosynthesis at greater than 30°C. In C_3 plants this is generally less than 25°C. Whereas C_4 plants are not usually saturated by light at intensities

below 50,000 lux, most C_3 plants are saturated at 30,000 lux. In their review Cooper and Tainton[35] suggest that all the properties of C_4 plants reflect little more than an adaptation to the tropical environment in which they are found.

(viii) Isotope discrimination

The rate of CO_2 fixation by RBPC proceeds at a slower rate with $C^{13}O_2$ than with $C^{12}O_2{}^{16}$. Thus C_3 plants (with only the Calvin cycle) were enriched with C^{12} relative to the atmosphere. PEPC does not discriminate between isotopes. Thus the ratio of C^{13}/C^{12} is higher in Crassulaceae and C_4 plants than in C_3 plants.

V. Diversity within C_4 plants

Considerable variation both in anatomical and biochemical features occur within the classification of C_4 plants. Hatch et al.[68] have divided C_4 plants into three groups: NADP ME, PCK and NADME types. Each is called after the major decarboxylating enzyme in each group. Thus, NADPME plants have a high level of NADP specific malic enzyme (EC 1.1.1.40) in its bundle sheath cells, the PCK PEP carboxy kinase (EC 4.1.1.49) and the NADME NAD specific malic enzyme (EC 1.1.1.38)

(i) The NADP ME types

These include plants such as maize, sorghum and pearl millet. These are monocotyledonous with the bundle sheath chloroplasts located at the edge of the sheath near to the mesophyll cells and distant from the vascular tissue. High levels of NADP specific malic enzyme and NADP specific malate dehydrogenase have been found in these chloroplasts[68].

However, in the dicotyledonous plant Gomphrene calosides (which is in this group), the chloroplasts are at the edge of the bundle sheath nearest the vascular tissue. The bundle sheath chloroplasts of both monocotyledonous and dicotyledonous plants of this group lack well developed grana.

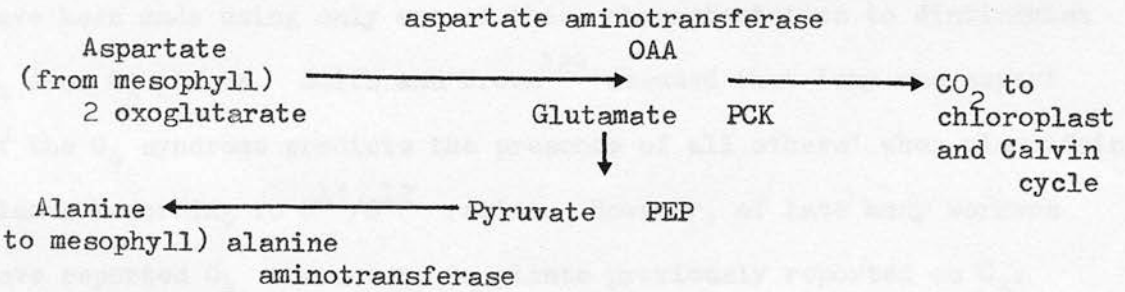
Malate, the major C_4 acid produced by photosynthesis in these plants is thought to migrate to the bundle sheath chloroplasts where decarboxylation takes place. The NADPH produced by the reaction is thought to reduce PGA, which results from Calvin cycle fixation of the CO_2 produced by decarboxylation of OAA. Pyruvate, the product of decarboxylation is thought to return to the mesophyll where it could be converted to PEP.

(ii) The PCK Type

These include the grasses Panicum maximum and Chloris gyane Apart from high levels of PEP carboxykinase in the bundle sheath, this type of plant also has high levels of the enzymes aspartate aminotransferase (EC 2.6.1.1.) and alanine aminotransferase (EC 2.6.1.2.) which appear to be equally distributed between the mesophyll and bundle sheath cells. The chloroplasts are distributed evenly throughout the bundle sheath cells, in contrast to the arrangement in NADPME plants.

Aspartate is the C_4 acid found in greatest concentration in these plants. The following pathway of carbon metabolism was proposed by Hatch et al.[68] for these plants:

Pathway of carbon in the bundle sheath cell of a PCK plant



In this scheme, aspartate, from the mesophyll cells is converted to OAA which is then decarboxylated to PEP by PCK. The CO_2 released is then converted to hexose via the Calvin cycle. Alanine, formed from pyruvate, derived from PEP, is then postulated to return to the mesophyll. The alanine is thought to be converted to PEP, in the mesophyll cells.

(iii) NADME Type

Plants of this type include saltbush, purslane, amaranth, and broomcorn millet. They produce aspartate as the major C_4 acid and the enzymes aspartate aminotransferase and alanine aminotransferase are present at high activities. Large numbers of mitochondria which appear to contain high levels of NAD specific malic enzyme, were found to be in close association with the bundle sheath chloroplasts. These were concentrated at the edge of the bundle sheath nearest the vascular tissue. Decarboxylation in this type of plant appears to occur in the mitochondria.

Gutierrez et al.[63] studying a rather larger population of plants, found a similar subdivision of C_4 plants to be appropriate. However, some species did not fit neatly into any of the three groups described by Hatch et al.[68]. It appears therefore that the diversity within C_4 plants is large

C_4 Character in C_3 plants.

Typical C_4 plants (e.g. maize, sugar cane, and sorghum), the leaves of which have all the characteristics mentioned previously will subsequently be referred to as classical C_4 plants. A number of surveys have been made using only one of these characteristics to distinguish C_4 from C_3 plants. Smith and Brown[124] assumed that 'any one aspect of the C_4 syndrome predicts the presence of all others' when classifying plants according to C^{13}/C^{12} ratios. However, of late many workers have reported C_4 characters in plants previously reported as C_3.

Khan and Tsunoda[82] found that certain types of wild wheats (C_3) adapted to dry conditions had leaf cells in a compact radiate arrangement round densely packed vascular bundles. This has some similarity to C_4 plant structure. The authors also showed the leaves of these wild type plants to have higher rates of photosynthesis than many

commercial varieties. The sunflower (C_3) shows rates of photosynthesis and translocation[74] comparable to those of C_4 plants.

A high activity of PEPC has been reported in the testa of pea[72]. Willmer and Johnston[146] have separated the products of $C^{14}O_2$ fixation of the skin of the developing tomato (C_3) and found them to be very similar to those of a C_4 plant.

Early in the development of the Antirrhinum leaf the level of PEPC is high[73]. Hedley and Rowland[73] put forward the hypothesis that the function of the enzyme was as an auxillarly CO_2 trap, as in the case of C_4 plants. However, it is equally probable that PEPC may be involved in protein synthesis (see Chapter 3).

High levels of PEPC have also been reported in the pericarps ofbarley[41] and wheat[148]. The levels of NADP specific malate dehydrogenase (EC 1.1.1.82) and NAD specific malate dehydrogenase (EC 1.1.1.37) were also estimated. The results were consistent with a C_4 photosynthesis of the NADME type[68]. An observation of particular interest is that the compensation point of the ear of wheat is lower than that of the leaves[43]. Thus, while PEPC activity is present in the leaves and tissues of a number of C_3 plants, its precise function is as yet not understood The evidence suggests that the immature cereal grain may have some characteristics of C_4 photosynthesis.

Conclusions

It has been shown that the classification of plants into C_3 and C_4 types on the basis of the pattern of photosynthesis of the leaves is not appropriate to all plants. For example, the tomato plant exhibits some of the properties of both, since it fixes CO_2 by the Calvin cycle alone in the leaves and by the C_4 pathway in the skin of the fruit. Furthermore many so called C_4 plants have been classified as such using only one of the range of C_4 characteristics e.g. isotope discrimi-

nation. Some C_3 plants have been shown to have some C_4 character.

A survey of plants for all properties associated with classical C_4 plants may well show that many have been incorrectly classified. It is also clear that other tissues apart from the leaves may be important in whole plant photosynthesis, as shown in studies of the outer layers of green tomato, pea testa and cereal ear. Thus, classification of C_3 and C_4 plants on the basis of leaf photosynthesis may not be justifiable.

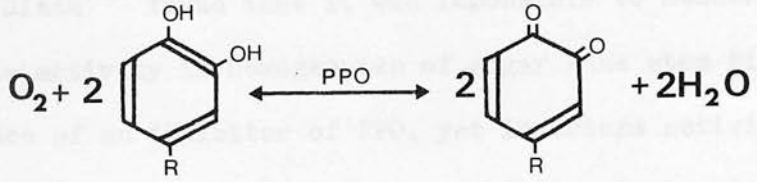
The evidence for the ear being a major photosynthetic organ is very strong. The pericarp has been shown to contain some of the enzymes responsible for photosynthesis. It contains chloroplasts and is uniquely located to trap CO_2 respired by the grain. There is little in the literature concerning the function of the pericarp yet evidence suggests that it may be associated with pathways of CO_2 fixation which differ from those of the leaves. The function of the immature cereal pericarp forms the subject of the present work.

Chapter 2

Phenols, Phenoloxidase and Inhibitors

Introduction

There are particular difficulties associated with the assay of plant cell constituents[86] due to the presence of phenols and the enzyme polyphenol oxidase (PPO) (EC 1.10.31). Thus in homogenates of plant cells the following reaction may occur.



orthodiphenol quinone

The oxidation of phenols may also occur non-enzymatically. In particular manganese has been shown to catalyse the above reaction[11]. The resultant quinone may react with a variety of cell constituents or polymerize to form brown coloured pigments. Such a browning reaction has been noted in homogenates of the barley pericarp[41]. Quinones react with amine, L-amino, imino and thiol groups. Reaction with such chemical groups present on the protein chain of an enzyme may lead to the inactivation of that enzyme[99]. Such reactions are thought to be involved in plant disease resistance[53]. Baldry et al.[10] claim that in intact cells of sugar cane leaves, PPO and its phenolic substrates are located in separate compartments, since gentle grinding of the leaves results in the release of phenols into the medium before PPO, indicating that the enzyme is located in an organelle more resistant to grinding. However, when a cell is damaged e.g. by infection, substrate and oxidase may come into contact and the polymer

formed may prevent further infection entering the cell. The inactivation of enzymes by quinone binding may both prevent metabolism by an invading organism and make host proteins unavailable to the parasite[114]. Useful though the phenol oxidase reaction may be *in vivo*, it makes the study of enzymes *in vitro* difficult. Not only are enzyme activities reduced by quinone attack, they are also differentially affected, i.e. enzymes show different susceptibilities to inactivation by quinones. Thus, Slack[120] found that it was impossible to measure sucrose synthetase activity in homogenates of sugar cane stem tissue without the presence of an inhibitor of PPO, yet invertase activity was only marginally increased by including inhibitor in the isolation medium.

The increase in activity of the enzymes with respect to the inhibitor is of interest. It could be concluded from the above that the effectiveness of the inhibitor was not as great for invertase as it was for sucrose synthetase. This may not be the case. Consider the assay of two plant enzymes; A and B, which are assayed in crude homogenates. The quinones produced in this preparation cause A to be inhibited by 50% and B by only 5%. Thus increase in activity caused by the effective inhibition of enzyme inactivation by quinones will be 100% for A and 5.3% for B. However, for both A and B the inhibitor of enzyme inactivation by quinone was 100% Since it is extremely difficult to estimate plant enzyme activity in the total absence of quinone, it is equally difficult to decide if an inhibitor of enzyme inactivation is equally effective for all enzymes.

Since it was important to establish the relative activities of various enzymes in the barley pericarp (Chapter 3) it was necessary to find a medium in which quinone inactivation of enzymes was kept to a minimum. As RBPC is known to be sensitive to quinone attack[78], its activity was used here as an indicator of enzyme inactivation by

quinone.

Various methods have been used to prevent the inactivation of enzymes by quinone.

I Addition of Protein to the Assay Medium.

Protein is not usually added to a medium prior to homogenisation as such protein rich solutions froth excessively. It is possible that by adding inert protein to the assay medium, quinone would be removed by binding to this rather than to the enzyme to be assayed. However, in a crude homogenate there is usually a large quantity of protein present, thus there is little likelihood of further protein addition being of any consequence. This method would also only be effective if the inert protein was more susceptible to quinone attack than the enzymes to be assayed.

II Addition of Polyvinyl Pyrrolidene (PVP)

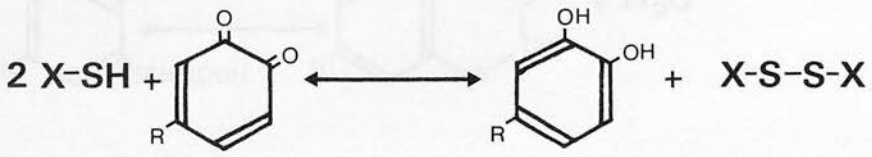
PVP has been effectively used to prevent quinone formation in cell and tissue homogenates[11] as it binds to phenols and makes them unavailable to PPO. PVP has a range of molecular weights and in the high molecular weight range it is insoluble in aqueous solution. The phenol PVP complexes may thus be removed by centrifugation. PVP does not however bind all phenols, the major exceptions being chlorogenic acid and catechin[142]. Chlorogenic acid has been found in many plant tissues and is a good substrate for PPO. Thus PVP is not entirely effective in preventing enzyme inhibition by the products of the PPO reaction.

III Addition of Thiol Compounds

Thiol compounds react with quinones and are often used to prevent the inactivation of enzymes by quinone[114]. Three different reactions may occur:

(i) Reduction of Quinone

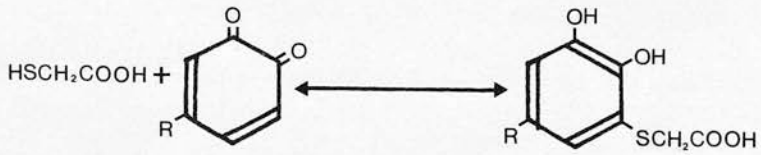
This reaction depends upon the relative standard oxidation-reduction potentials of the quinone and the thiol. Thus a careful choice of thiol must be made if all quinones are to be removed by this method. In this case phenols are oxidised to quinones by PPO and then reduced back to phenols by thiol. The net reactants are oxygen and thiol, The products oxidised thiol and water.



The action of PPO is thus reversed establishing a cycle of phenol oxidation and quinone reduction. Ascorbate has been used to reduce quinones to phenols as above and has been shown to prevent the formation of a brown precipitate[130].

(ii) Subsitution of the aromatic ring.

This occurs when there is no steric hindrance. Thus in the case of thioglycollate the following reaction occurs:

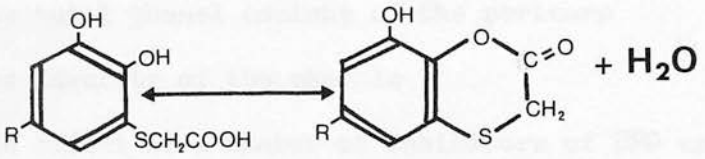


The product is a substituted phenol and is a suitable substrate for PPO. Thus a succession of quinone substitution and phenol oxidation

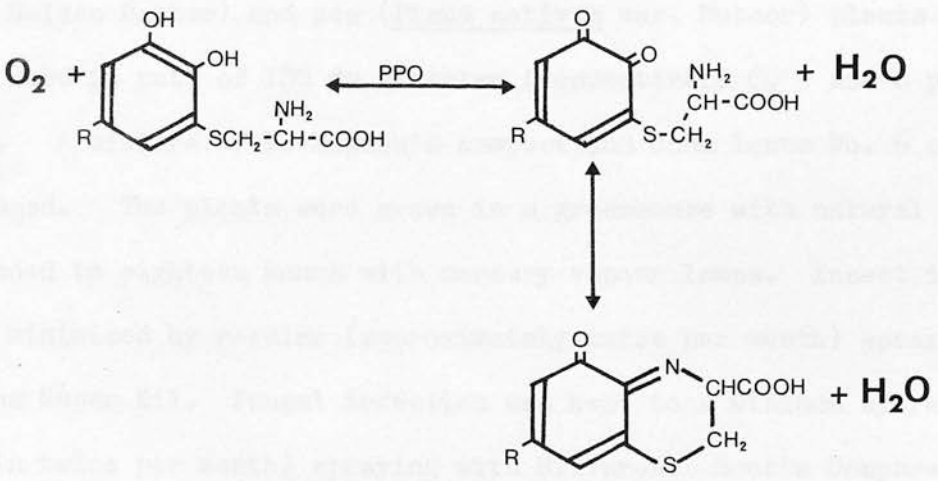
reactions may take place until all the hydrogen atoms on the six carbon ring have been substituted.

(iii) Condensation with the carbonyl group.

This reaction takes place with certain thiol groups after the above substitution occurs. Thus, with the monosubstituted diphenol of thioglycollate the following reaction occurs:-



And, with cysteine monosubstituted diphenol:-



The products of such reactions are not available for further reaction with PPO as is the case after substitution and reduction. Thus thiol is not consumed rapidly yet a high concentration is required for the reaction to proceed. Coombs et al.[34] found that one of the

enzymes which was inactivated by quinones was PPO itself. This inactivation was most effectively prevented by the addition of thioglycollate to the medium.

Thus, in order to discover the most effective method of preventing enzyme inactivation by quinones, the following experiments were devised to determine:-

1. The activity of PPO in homogenates of pericarp tissue
2. The total phenol content of the pericarp
3. The identity of the phenols
4. The effect of a number of inhibitors of PPO upon the activity of RBPC and PEPC

Methods

I Plant material.

Two row barley (Hordeum distichum var. Julia) maize (Zea mays var. Golden Bantam) and pea (Pisum sativum var. Meteor) plants were grown from seed in pots of 180 mm diameter (respectively 6, 3 and 6 plants per pot). A mixture of Levington's compost and John Innes No. 6 compost was used. The plants were grown in a greenhouse with natural daylight extended to eighteen hours with mercury vapour lamps. Insect infestations were minimised by regular (approximately twice per month) spraying with Fisons Super Kil. Fungal infection was kept to a minimum by regular (again twice per month) spraying with Milfaron. Boot's Compure was used to maintain nutrient levels in the soil. Insecticide, fungicide and fertilizer were used throughout according to manufacturers' directions. Sedum spectabile once established was maintained in these conditions.

Grain was taken from the barley plant 25-30 days after anthesis immediately prior to use. The husk and transparent layer of the pericarp were peeled from the grain with forceps. The green pericarp tissue

was then removed by gently scraping the grain with a scalpel, and immediately placed in buffered medium or in water (both at 4°C)

II Estimation of PPO Activity in Barley Pericarp Tissue.

The method of assay was essentially that of Baldry et al.[11]. Twenty pericarps were placed in 4.0 ml of buffered medium (50 mM-tricine KOH pH 7.5, 0.33 M-sorbitol) and homogenised by hand in a Griffiths' all glass tissue grinder. The homogenate was filtered through four layers of muslin. The increased rate of uptake of oxygen by homogenates of pericarp tissue when supplied with phenolic substrate was measured polarographically. A sample (3.0 ml) of filtrate was placed in the chamber of a Rank oxygen electrode jacketed with a circulating supply of water at 30°C, calibrated by the method of Walker et al.[141]. The chart recorder was set at zero when the chamber of the oxygen electrode contained a freshly prepared solution of sodium dithionite. The potentiometer of the electrode was then adjusted to read 0.7 full scale deflection when the chamber contained 3.0 ml of air saturated water, (1.0 l of air saturated water contains 5.3 ml of oxygen at 30°C[80]) so that the full scale deflection of the chart recorder was equivalent to the chamber holding 1.0 µmol - O_2. The zero and 0.70 were set twice more to ensure that adjustment of the potentiometer had not affected the zero reading.

The filtered homogenate was left for about one minute to warm and equilibrate with the atmosphere. The cap was placed on the chamber and the rate of oxygen uptake recorded on a chart recorder. A sample (0.05 ml) of substrate was added and the subsequent rate of oxygen uptake recorded. The amounts of substrate added were 2.0 µmols of adrenalin, catechol, orcinol, phenylalanine, phloroglucinol, and L-tyrosine, and for the following less soluble compounds 1.0 µm of caffeic acid, chlorogenicacid, p coumaric acid and quercitin. The

experiment was repeated using buffer instead of homogenate to estimate the rate of oxygen uptake by these compounds in the absence of homogenate.

The rate of phenol oxidation was expressed as the rate of oxygen uptake of the pericarp, homogenate and substrate (a) minus the sum of the rate of oxygen uptake by the homogenate supplied with no substrate (b) and the rate of substrate oxidation without homogenate (c) i.e. a - (b + c). The rate was expressed in nmol - O_2 min^{-1} for the sample, which was fifteen pericarps.

III Estimation of the Amount of Phenol in the Pericarp of Barley and the Leaves of Peas Sedum and Maize.

A green coloured complex is formed when phenols are added to solutions of ferric chloride[79]. A ferric chloride-chlorogenic acid complex was found to have an absorption maximum at 720 nm. Homogenates of barley pericarp (5/ml) and leaves of maize, pea and Sedum were prepared in water at 4°C. Samples (1.0ml) of each was placed in a cuvette to which was added 2.0 ml 0.02% (w/v) ferric chloride solution. The optical density at 720 nm was measured. The optical density of a homogenate prepared as above, but with water substituted for ferric chloride, was substracted from the previous figure to allow for the homogenate absorption at this wave length. A standard curve of the optical density at 720 nm of ferric chloride/chlorogenic acid complex in the rate of 0 - 1.5 nmol per sample chlorogenic acid was prepared. The ferric chloride - phenol complexes of the homogenates were assumed to have similar spectroscopic properties to the ferric chloride - chlorogenic acid complex. The standard curve was used to convert optical density of the ferric chloride-phenol complexes of homogenates into chlorogenic acid equivalents.

The chlorophyll content of each homogenate was determined by the

method of Arnon[6]. Results were expressed as μmol chlorogenic acid equivalents per mg chlorophyll.

A homogenate of each tissue was prepared in a medium of 0.33 M-sorbitol, 50 mM-tricine KOH buffer at pH 7.5. A sample was used for chlorophyll determination[6] and another for protein determination by the method of Lowry et al.[96]. The ratio of protein to chlorophyll was determined for each tissue. This ratio was used to determine the chlorogenic acid equivalents in the tissue on a per mg protein basis.

IV Idenitification of Phenols in the Pericarp of Barley.

Pericarps from grain at various stages of development were homogenised in 50% acetone in water (10 per ml). The 1,500 g supernatant was subjected to paper chromatography in one dimension[113]. Samples of chlorogenic acid, p coumaric acid and caffeic acid were run simultaneously. The developer was prepared by mixing equal quantities of butanol:water (370:25) and proprionic acid :water (18:22). The chromatogram was run for 24 hours and then dried (approximately 80°C). Phenolic compounds were identified by fluorescence under ultra violet light.

V Effect of Inhibitors of Quinone Formation upon the Activity of RBPC from the Barley Pericarp.

RBPC activity was measured by a method similar to that of Slack and Hatch[121]. The incorporation of $C^{14}O_2$ into acid stable compounds was determined in homogenates supplied with ATP and ribose 5 - phosphate(R5P) Pericarp homogenates (10 ml) were prepared in a medium of 0.33 M - sorbitol, 50 mM - tricine KOH buffer pH 7.5, 1mM-$MnCl_2$, 1mM-$MgCl_2$, and 1 mM inhibitor, either ascorbate, cysteine, dithiothrietol, glutathione, or mercaptoethanol. The homogenates were filtered through four layers of muslin. Samples (0.2 ml) of homogenate were placed in

small test tubes to which were added 0.1 ml of the buffered medium containing different amounts of inhibitor to bring the final concentration to that required in the experiment. To each tube was also added 0.03 ml of 0.75 M-sodium (C^{14}) bicarbonate (specific activity 0.33 mCi/mmol). The tubes were then placed in a water bath at 30°C and left for five minutes to equilibrate. The carboxylase reaction was started by adding 0.06 ml of the buffered medium containing 0.6 µmol ATP and 0.6 µmol R5P. Samples (0.1ml) were withdrawn immediately and after 3.0 minutes and 6.0 minutes and the reaction stopped by adding 0.5 ml of 5% (w/v) trichloroacetic acid (TCA) in ethanol.

The ethanolic mixtures were then heated in a water bath at 70°C for 30 minutes to drive off unfixed $C^{14}O_2$. After centrifugation at 5,000 g for 10 minutes to remove protein precipitate, the supernatants were added to scintillation vials containing 5.0 ml of a dioxan based scintillant (50 g napthaline and 6 g 2, 5 diphenyloxazide per 1 l 1,4 dioxan) and the samples counted for 20 minutes in a Beckman scintillation counter (counting efficiency 70%).

VI Oxygen Uptake by Inhibitors of Quinone Formation.

Samples (2.7 ml) of the buffered medium as used for the determination of RBPC activity, but without inhibitor were added to the chamber of an oxygen electrode. Once equilibrium had been established 0.3 ml of 0.1 M inhibitor was added, and the rate of oxygen uptake recorded on a chart recorder as described previously.

VII The Effect on PEPC Activity by Compounds used to Prevent Inactivation by Quinones.

(i) The procedure was similar to that used for the determination of RBPC activity. After addition of sodium (C^{14}) bicarbonate, 0.03 ml of the buffered medium containing 2.4 µmol of sodium glutamate was

added to each tube. The tubes were then placed in a water bath at 30°C and left to equilibrate for 5 minutes. The reaction was initiated by the addition of 0.03 ml of buffered medium containing 1μmol of PEP. Samples (0.1 ml) were withdrawn immediately and after 1.5 minutes and 3.0 minutes and the reaction stopped by placing in 0.5 ml 5% TCA (w/v) in ethanol. The amount of radioactivity present in the ethanolic mixtures was determined as described above.

(ii) Pericarp homogenates were prepared as described previously for the determination of RBPC activity, except that the medium contained either 10 mM-cysteine, 10 mM-mercaptoethanol, 100 mM-mercaptoethanol or 0.2% (w/v) bovine serum ablumin. The activity of PEPC was measured as above.

Results

I Activity of PPO in Barley Pericarp Tissue

Of the compounds used only adrenalin took up oxygen in the absence of added homogenate. The activities of PPO in homogenates of barley pericarp supplied with various substrates are given in Table 2.1. The highest rate of oxidation was observed with adrenalin, much higher than any other. The rate of oxidation with orcinol was barely significant. Catechol, L-phenylalanine, phloroglucinol and L-tyrosine were not oxidised.

II Phenol content in Barley Pericaps and Leaves of Pea, Maize and Sedum

The standard curve of optical density against quantity of chlorogenic acid added to ferric chloride solution is shown in Fig. 2.1. The amounts of chlorogenic acid equivalents found in each of the tissues are shown in Table 2.2. The barley pericarps contained the highest amount of phenol both on a chlorophyll and on a protein basis. Barley pericarps contained more than five times the amount of phenols on a mg chlorophyll basis than that of maize.

Table 2.1

Rates of Oxygen Uptake by Pericarps Supplied with Various Phenolic Substrates

Substrate	nmol O_2/min/15 Pericarps
Adrenalin	55.0 ± 20.0
Caffeic Acid	6.5 ± 1.0
Catechol	0
Chlorogenic Acid	10.0 ± 1.0
p - Coumaric Acid	14 ± 1.0
Orcinol	1.0 ± 0.7
L - Phenylalanine	0
Phloroglucinol	0
Quercitin	40 ± 1.0
L - Tyrosine	0

Figures are the mean of three estimates ± standard deviation

Figure 2.1 Absorption by Ferric Chloride – Chlorogenic Acid Complex at 720 nm.

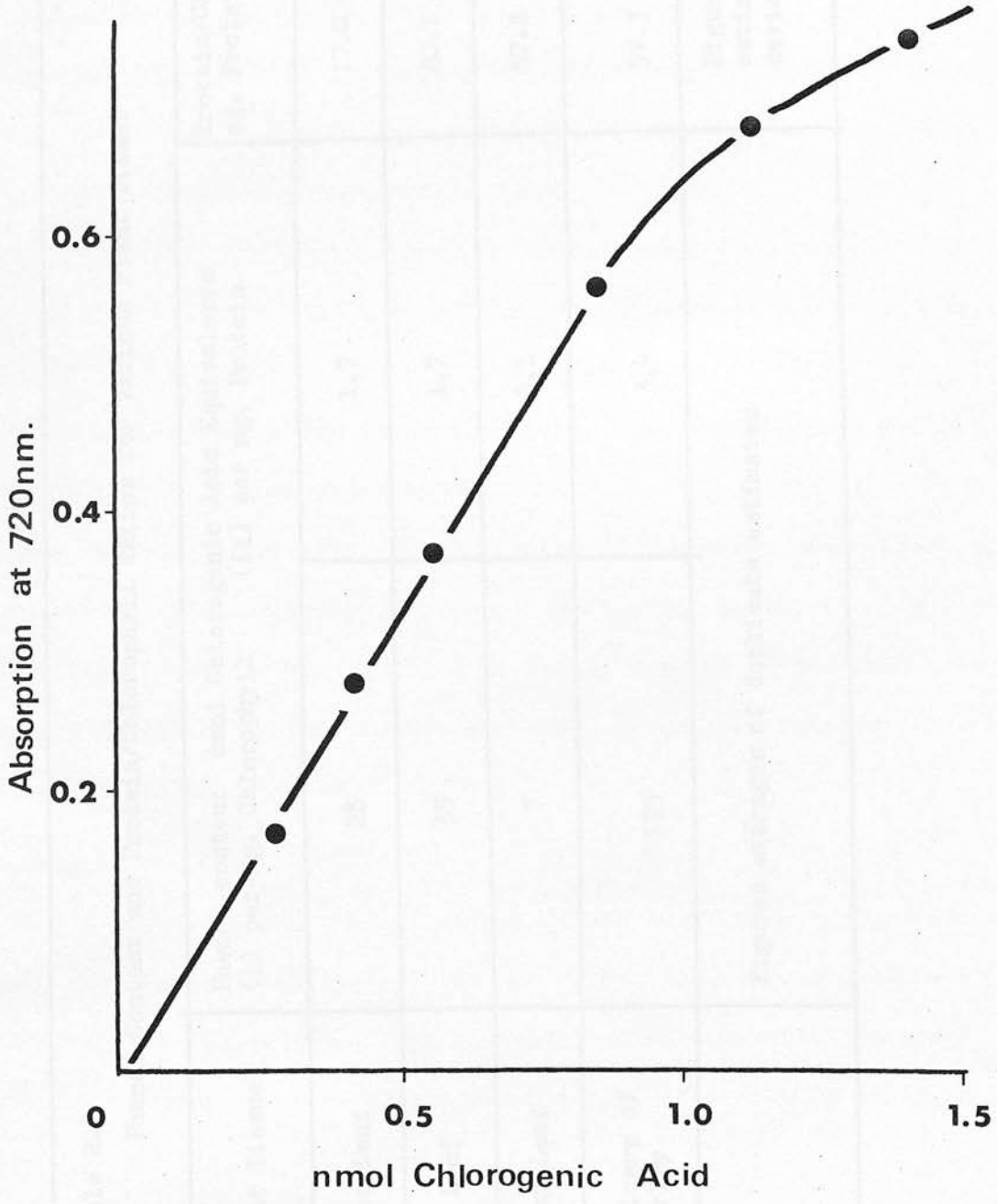


Table 2.2

Phenol Content and Protein/Chlorophyll Ratios for Various Plant Tissue

Plant Tissue	Phenol content umol Chlorogenic Acid Equivalents (i) per mg. Chlorophyll	(ii) per mg. Protein	Protein/Chlorophyll Ratio mg. Protein/mg. Chlorophyll
Maize Leaf	28	1.7	17.0 ± 0.4
Pea Leaf	35	1.7	20.1 ± 3.4
Sedum Leaf	7	0.1	57.2 ± 7.6
Pericarp of Barley	195	3.4	57.3 ± 0.9
	Figures averages of duplicate estimates		Figures averages of three estimates ± standard deviation.

III Identification of Phenols.

The standard phenolic compounds were easily identified under ultraviolet light, however, no compounds were detected in homogenates of pericarp except for a pink-brown compound which had a Rf of 0.36, from grain 48 and 50 days after anthesis. No such compound was identified in younger grain.

IV Effect of Thiols and Ascorbate on RBPC activity

The activity of RBPC at various concentrations of inhibitor is shown in Fig. 2.2. RBPC activity was practically independent of the concentration of ascorbate and glutathione used.

RBPC activity ($C^{14}O_2$ fixation) rates increased dramatically with concentration of cysteine, mercaptoethanol and dithiothreitol in the range of 10^{-6} M - 10^{-2} M. At higher concentrations of thiol RBPC activity was reduced. The highest activity was measured in a medium containing 10^{-2} M - mercaptoethanol.

For the sake of clarity, no indication of the error associated with estimation of RBPC activity is shown. Each point is the mean of four estimations. The average standard deviation was 17% of the mean (the standard deviation of this mean was $\pm$ 14%). Error was greatest in estimation of low rates of $C^{14}O_2$ fixation.

V Oxygen uptake of 10 mM Solution of Inhibitor

Table 2.3 shows the rate of oxygen uptake by 10 mM solutions of thiols and ascorbate which were the inhibitors used in the study of RBPC Activity. Oxidation of the different thiol compounds proceeded at similar rates, while ascorbate was oxidised at a notably faster rate.

VI Variation in PEPC Activity with Mercaptoethanol Dithiothreitol and Cysteine Added after Homogenisation.

The response of PEPC to dithiothreitol and to mercaptoethanol was

Figure 2.2 Effect of Various Compounds upon the Activity of RBPC

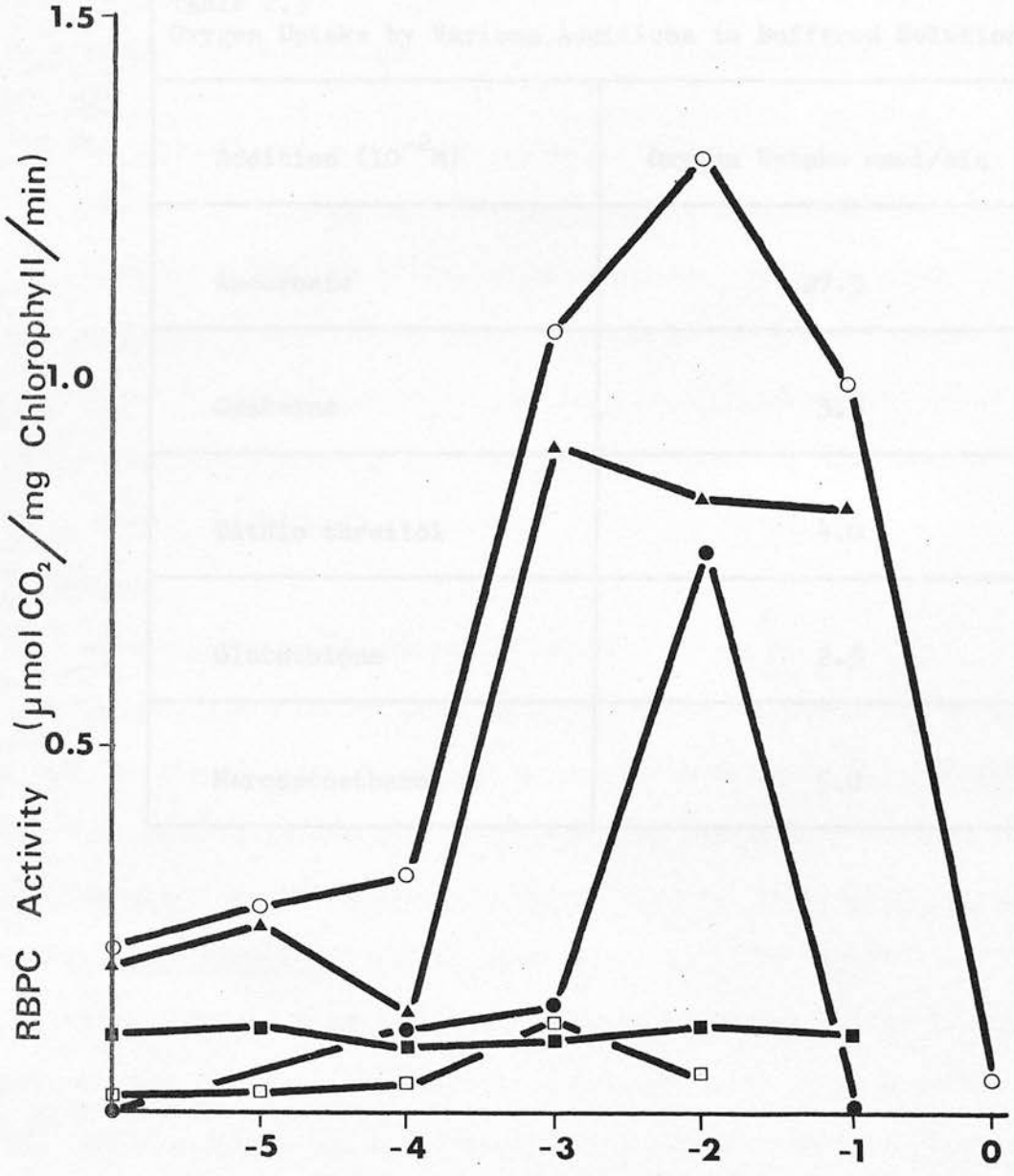


Table 2.3
Oxygen Uptake by Various Additions to Buffered Solution

Addition (10^{-2}M)	Oxygen Uptake nmol/min
Ascorbate	27.5
Cysteine	3.5
Dithio threitol	4.0
Glutathione	2.5
Mercaptoethanol	5.0

very similar (Fig. 2.3). PEPC activity was substantially constant over the range 10^{-6} - 10^{-2}M. However, activity was almost doubled at 10^{-1}M. Higher values of concentration could not be obtained because of problems involved with the solubility of cysteine. Over the range 10^{-6} - 10^{-3} M cysteine the activity of PEPC was approximately twice that of the medium containing dithiothreitol or mercaptoethanol.

VII Response of PEPC Activity to Homogenisation in Cysteine Mercaptoethanol and Bovine Serum Albumin (Table 2.4)

The lowest activity of PEPC was found in samples to which no compound was added to inhibit inactivation by quinones. PEPC activity was greater in 10^{-1} M-mercaptoethanol than in 10^{-2}M, but not significantly so. In 10 mM-cysteine activity was less than in 10 mM-mercaptoethanol with bovine serum albumin activity was approximately the same as that in 10 mM-mercaptoethanol.

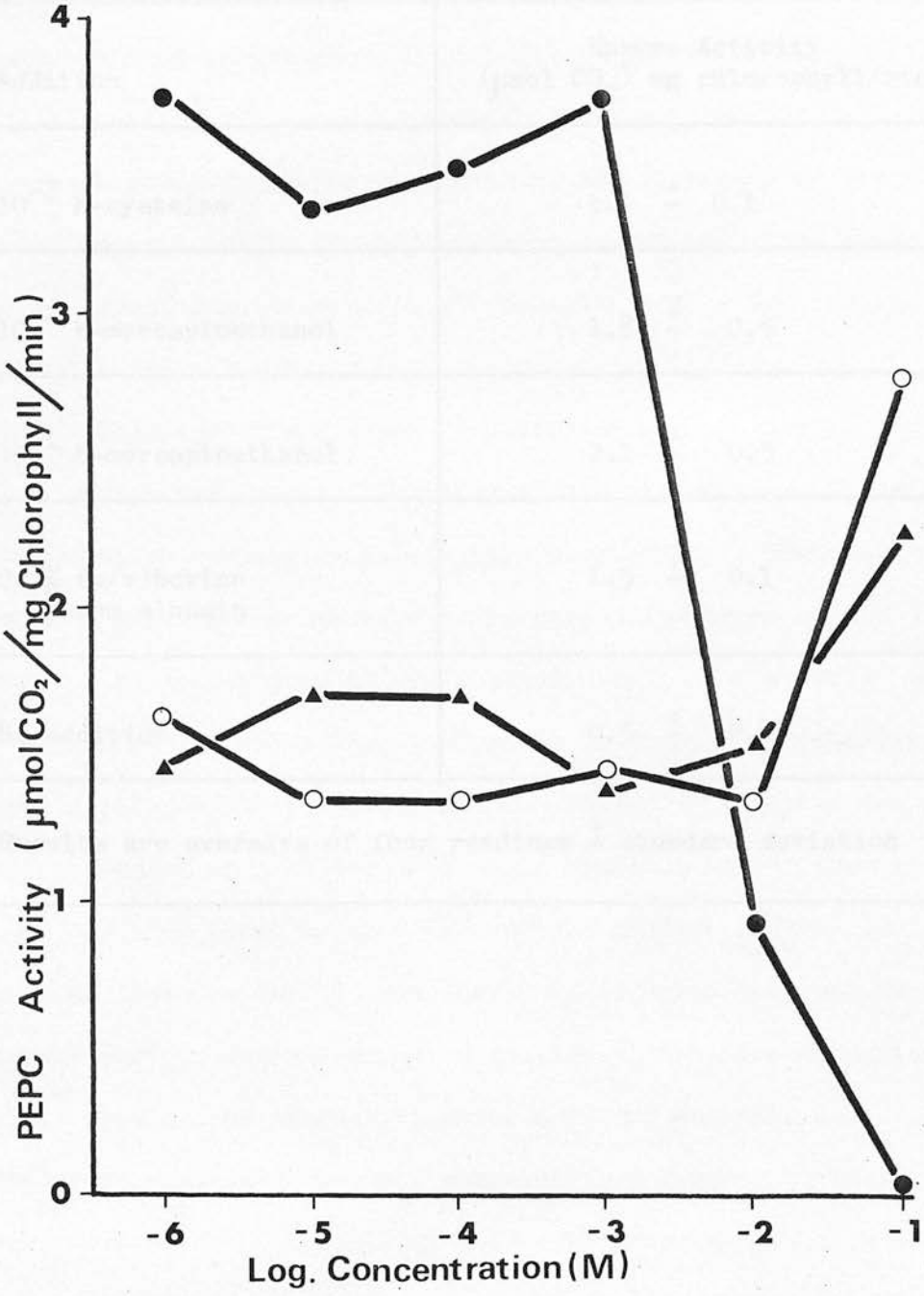
Discussion

I Low Activity of Polyphenol Oxidase.

Chlorogenic acid has been shown to be the substrate of PPO that gives the highest rate of oxygen uptake in some plants[11]. The rate of chlorogenic acid oxidation reported here was similar to that reported by Ku et al.[87] in the leaves of Panicum texanum, Pennisetum purpureum and Spinacia oleracea. These plants were noted for their low PPO activity. Adrenalin gave the highest rate of oxygen uptake in pericarp tissues. Since adrenalin is a primary amine it is possible that the pericarp enzyme has a requirement for such a grouping. Such a polyamine oxidase has been found in barley in sufficient amounts to permit its characterisation[125]. The activity of the enzyme from the pericarp with adrenalin as substrate was, however, much lower than the chlorogenic acid oxidase activity found in many plant tissues[87].

It is difficult to reconcile the low activity of PPO reported here

Figure 2.3 Effect of Thiol Concentration upon the Activity of PEPC



● cysteine, ▲ dithiothreitol, ○ mercaptoethanol

Table 2.4
Activity of PEPC from Barley Pericarps Homogenised in Buffered Media containing various Additions

Addition	Enzyme Activity (μmol CO_2) mg chlorophyll/min
10^{-2} M-cysteine	1.1 $\pm$ 0.1
10^{-2} M-mercaptoethanol	1.8 $\pm$ 0.5
10^{-1} M-mercaptoethanol	2.1 $\pm$ 0.3
0.2% (w/v)bovine serum albumin	1.5 $\pm$ 0.1
No addition	0.5 $\pm$ 0.4

Results are averages of four readings $\pm$ standard deviation

with the observation that homogenates of pericarp tissue brown easily[41]. PPO has been shown to have a vast range of substrates, only a few of which havebeen tested here. It is possible that the substrate for which the enzyme is specific (i.e. the physiological substrate) was not among them. This substrate is presumably present in the pericarp or an adjacent tissue.

In sugar cane homogenates, PPO itself is inactivated by quinones[34]. A similar reaction may occur in pericarp homogenates. The chlorogenic acid oxidase activity of the pericarp was not increased by including 10 mM-mercaptoethanol in the homogenising medium, yet this prevented browning. Thus, inactivation of PPO by quinone does not explain the low level of PPO activity.

II Phenol/Chlorophyll and Phenol/Protein Ratios

When expressing results variables should be expressed in relation to a constant or unchanging parameter. Chlorophyll is frequently chosen as a basis for comparison. However, comparisons on a chlorophyll basis may lead to wrong conclusions. For example, the chloroplasts of the maize bundle sheath lack grana[68] and therefore may have a reduced chlorophyll content. Thus, for example, phenol content expressed on a chlorophyll basis would be higher than if expressed by some other means. Chlorophyll content changes during development (see Chapter 5), and therefore is not a good standard for comparison[41]. Protein may also provide a basis for comparison, but again specialised tissues like the pericarp may have an atypically high protein content, and therefore results would be low. However, both on a per mg chlorophyll basis and on a per mg protein basis the pericarp contains much more phenol (chlorogenic acid equivalents) than the leaf tissues. The possibility of non-enzymic oxidation must not be excluded. The browning reaction observed

in pericarp tissue may be due to polymerisation of quinone produced by a metal ioncatalysed chemical reaction involving oxygen and phenol.

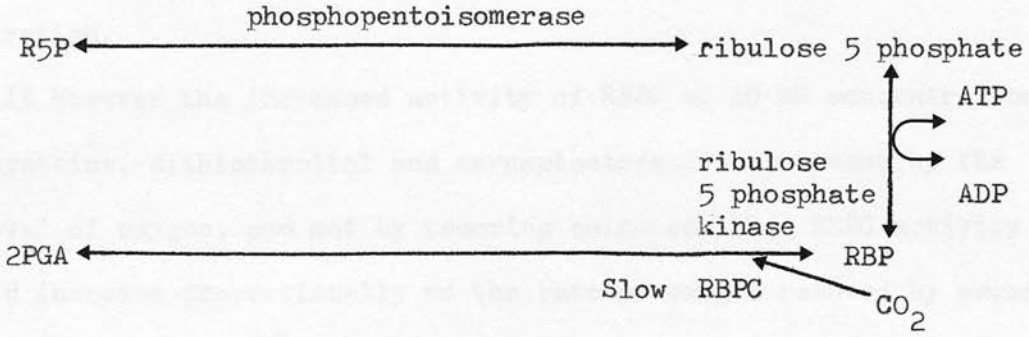
III Inadequacy of the Method of Phenol Separation

The method used for the identification of the phenols present in pericarp tissue was not adequately sensitive to detect the phenol recovered from the small amount of tissue that was available.

The pink-brown compound identified in pericarps of grain about 50 days after anthesis was probably the same as that noticed by Archbold[4]. The compound was formed to a greater extent in ears that were shaded. She concluded that this compound was not anthocyanin because of its solubility characteristics in water and alcohol.

IV RBPC Activity and Thiol Concentration

If it is assumed that the only effect of the inhibitors used was to prevent quinone inactivation of RBPC, it is apparent from Fig 2.2 that the most effective compound was mercaptoethanol. The decline in activity of RBPC at high concentrations of inhibitor cannot be explained as an effect upon quinone. At the high concentrations of mercaptoethenol, dithiothreitol and cysteine, the thiol compounds were probably directly affecting one of the enzymes involved in the assay of PBPC. There are three enzymes involved:-



Disulphide bridges stabilise the conformation of proteins. These bridges may be reduced by a high concentration of thiol[77] resulting in loss of enzyme activity. Inactivation of one of the three enzymes

by this mechanism may be responsible for the low $C^{14}O_2$ fixation at high concentrations of thiol.

V Possible Effect of Oxygen Removed by PPO

Baldry et al.[9] studied the effects of various thiols on the activity of RBPC from sugar cane. There are some notable discrepancies between these results and those reported here. Baldry et al.[9] found that cysteine had no effect upon the activity of RBPC while glutathione increased the activity of the enzyme. In the present work, in homogenates of pericarp, cysteine increased the rate of $C^{14}O_2$ fixation, while glutathione did not increase activity of RBPC to any significant extent. The concentration of mercaptoethanol which gave maximum activity of RBPC in preparations of sugar cane was one hundred times less than in the homogenates of barley. This, no doubt reflects the difference in phenol content of the two tissues. Since Mn^{2+} (manganese ion) can catalyse direct oxidation of thiol compounds it is possible that the oxygen concentration is reduced in the medium by this mechanism. Thus, since oxygen is an inhibitor of RBPC activity[24], the rate of CO_2 fixation may be increased. However, in the present experiment, the rate of oxygen uptake by the compounds used was not high and therefore oxygen deprivation is unlikely to be the cause of RBPC activation.

If however the increased activity of RBPC at 10 mM concentration of cysteine, dithiothreitol and mercaptoethenol, was caused by the removal of oxygen, and not by removing quinones, then RBPC activity would increase proportionally to the rate of oxygen removed by ascorbate and thiols. Ascorbate showed the highest rate of oxygen uptake, yet did not increase RBPC activity. Of the thiol compounds used mercaptoethanol showed the highest oxygen uptake and the highest RBPC activity followed by dithiothreitol cysteine and then glutathione.

On the other hand, if ascorbate has such a high oxygen uptake that all the ascorbate was oxidised prior to estimation of RBPC then a low activity of RBPC would be expected and the theory of oxygen uptake activating RBPC might still be a valid one. However, if the oxygen diffusion from the atmosphere to the medium was fast enough to maintain the initial oxidation rate of ascorbate, then the 10 mM solution would last 16 hours, much longer than the course of the experiment. Increased rates of $C^{14}O_2$ (14-carbondioxide) fixation in solutions of mercaptoethanol, cysteine and dithiothreitol, as observed, are thus on balance unlikely to result from a decrease in oxygen concentration of the medium.

VI Effect of Thiols on PEPC

If the effect of the thiols was solely to remove quinones from the medium the relative increase in enzyme activity would be similar for all enzymes inhibited by quinones. However, the effects of the thiols on RBPC and PEPC were different. There was no inactivation of PEPC at the highest concentration of mercaptoethanol and dithiothreitol as was observed with RBPC. The rates of $C^{14}O_2$ fixation by PEPC in the presence of 10^{-6} - 10^{-3} M cysteine were twice those observed in dithiothreitol and mercaptoethanol in the same concentration range. Cysteine, in the above concentration range increased the activity of PEPC more than RBPC.

PEPC may be sensitive to a particular quinone, the oxidation reduction potential of which makes it more susceptible to reduction by cysteine than other thiols.

However, there is no evidence to support this view as the phenols of the pericarp were not characterised. There is no general theory which accounts for the particular effects of individual thiols[114].

VII Addition of Thiol after Homogenisation.

The graphs of RBPC and PEPC activity against inhibitor concentration (Fig. 2.2 and 2.3) were prepared from results obtained by adding inhibitor after homogenisation of the pericarp tissue. However it was possible that the reaction between enzymes and quinones occurred most rapidly during homogenisation when phenol and PPO come in contact. The activities of PEPC estimated in 10 and 100 mM - mercaptoethanol and 10 mM -cysteine added prior to homogenisation were not significantly different from those obtained when the inhibitors were added after homogenisation (Fig. 2.3) It would thus appear that the production of quinones probably occurs some time after the mixing of phenol and PPO.

It has been suggested by Kenten[81] that PPO exists within the cell as a proenzyme or bound to an inhibitor. PPO then has activity only after activation. In Kenten's experiments the PPO of broad bean leaves required treatment with acid or alkali, or incubation with ammonium sulphate before maximum activity of the enzyme could be detected[81]. Thus it is possible that during homogenisation of pericarp tissue no added inhibitor of PPO is required because it is only upon subsequent incubation that the products of PPO inhibit RBPC or PEPC.

Conclusion

It is clear that the forumulation of a suitable medium for enzyme extraction and assay from the barley pericarp poses a number of problems. The high phenol content of the tissue appears to be a cause of low enzyme activities. A universally appropriate medium is required. A medium containing 10^{-2} M-mercaptoethanol resulted in the greatest activity of RBPC yet PEPC activity was highest in a medium containing 10^{-6} - 10^{-3} M-cysteine. The response of PEPC activity to cysteine was so different from that of the other thiols that choice of a cysteine

containing medium for later experiments may lead to atypical results. The theoretical advantage of cysteine condensing with the quinone carbonyl group[99] appeared to be of little practical importance, a conclusion also reached by Anderson[2].

A medium containing 10^{-2} M-mercaptoethanol was selected as most likely to lead to minimal enzyme inactivation by quinones in pericarp homogenates.

Chapter 3

Enzymology of the Barley Pericarp

Introduction

It was found by Slack and Hatch[121] that the enzyme complement of the leaves of C_4 plants differed from that of C_3 plants. Thus it was suggested by Chang and Troughton[30] that C_4 plants could be identified by the enzyme complement of their leaves. However, there are many areas of uncertainty regarding the enzyme complement of C_4 plants.

I Phosphoenol Pyruvate Carboxylase (PEPC), Ribulose 1,5, Bisphosphate Carboxylase (RBPC) and Glycollate Oxidase (GO)

The initial experiments of Slack and Hatch[121] indicated that on a chlorophyll basis the activity of PEPC was much greater in C_4 leaves than C_3 leaves, but that of RBPC was greater in the leaves of C_3 plants. Ku *et al.*[87] have found that most of the RBPC of C_4 plants is located within the bundle sheaths. As the bundle sheaths are more resistant to grinding than the mesophyll cells it is possible that the low level of RBPC reported by Slack and Hatch[121] is a consequence of incomplete homogenisation of the leaf[20]. Alternatively such a result could be explained by quinone inactivation of RBPC[26] (see also Chapter 2).

Photorespiration is a property of C_3 plants (see Chapter 1) and the bundle sheath cells of C_4 plants resemble cells from C_3 plants[87]. Therefore, if photorespiration occurs in C_4 plants it is likely to take place within the bundle sheath. Thus GO (EC 1.1.3.1.) an enzyme responsible for as much as half the light dependent oxygen consumption (i.e. photorespiration)[135] might also be expected to be concentrated within the bundle sheath. The observations of Tolbert *et al.*[136] that the activity of GO in C_4 plant leaves was less than that observed C_3 plant leaves, could again be explained by incomplete homogenisation of the bundle sheath.

II Chlorophyll a: Chlorophyll b Ratios

Many workers have shown that the ratio of chlorophyll a: chlorophyll b is higher in the leaves of C_4 plants than in the leaves of C_3 plants [23,30,75]. It was suggested by Black and Mayne[23] that this was due to the lack of chlorophyll b in the bundle sheath cells of NADP-ME type of C_4 plants (see chapter 1). However Holden[73] showed that C_4 plants of the other groups also had high chlorophyll a: chlorophyll b ratios.

III Formation of Phosphoenol Pyruvate (PEP)

The precise origin of the acceptor (PEP) in C_4 plants and the nature of the enzymic mechanism involved in its sythesis has not yet been established and may indeed vary within and between species. PEP may be formed by the catabolism of hexose phosphate. This would be a wasteful process as hexose phosphate is a precursor of sucrose and starch. The following two enzymes have been separately implicated in PEP synthesis.

(i) PEP Synthetase

Pyruvate phosphate dikinase (PEP synthetase) catalyses the following reaction[70]:

$$\text{Pyruvate} + \text{ATP} + \text{Pi} \longleftrightarrow \text{PEP} + \text{AMP} + \text{PPi}$$

and appears to be the enzyme responsible for regeneration of PEP in C_4 plants.

(ii) PEP Carboxy-kinase (EC 4.1.1.49)

This enzyme was proposed by Edwards et al.[45] to be responsible for PEP formation, and decarboxylation of oxaloacetic acid (OAA). The reaction is:

$$\text{OAA} + \text{ATP} \longleftrightarrow \text{PEP} + \text{CO}_2 + \text{ADP}$$

In this reaction only one of the ATP Phosphate groups is hydrolysed thus indicating a lower energy requirement than the PEP synthetase

reaction. However, this reaction is unlikely to be the source of PEP in C_4 plants as the enzyme appears to be located in the bundle sheath[45], therefore it would be necessary to postulate a migration of PEP, either by passive diffusion or by an active transport process, to the mesophyll layer where the carboxylation reaction is thought to occur

The reaction still has a possible place in PEP formation if it is assumed that there is no specialisation in the bundle sheath cells. If the Calvin cycle is located in the mesophyll chloroplasts and PEPC in the cytoplasm, as was suggested by Bucke and Long[26], the PEP formed by the carboxykinase reaction would only have to diffuse through the chloroplast membranes to arrive at the site of PEPC action.

IV The C_4 Leaf

In general it would appear that a 'typical' C_4 leaf should have at least the following properties:

a) high PEPC activity (on a chlorophyll basis)

b) low activities of RBPC and GO (but see page 75)

c) a high chlorophyll a : chlorophyll b ratio

d) a mechanism for the regeneration of PEP

The present wotk is an attempt to assess these characteristics both in plant leaves, representing each class of CO_2 fixation i.e. pea (C_3), maize (C_4) and in the barley pericarp. It was hoped that the pattern of enzyme activity in the barley pericarp would be similar to one of the leaves investigated, thus giving a good indication of the nature of carbon dioxide fixation in pericarp tissue.

Methods

I Plant Material

Plants were grown as described in Chapter 2. Pericarps from grain taken 25 days after anthesis from Hordeum distichum var. Julia were used for enzyme assays because the chlorophyll content of the pericarp is

then at its maximum value[41]. Enzyme activities may also be expected to be maximal at this time.

Grains from the other cereals, Hordeum hexastichum var. Sen tor, Scottish Bere, Hordeum vulgare var. Albino Lemma, Triticum aestivum var. Maris Dove, and Avena sativa var. Astor, was taken when morphology was similar to that of the grain of Julia 25 days after anthesis. Albino Lemma, a mutant barley[127], the pericarps of which are not capable of photosynthesis due to the absence of chlorophyll, was included as a control.

Grain from Julia was dissected and homogenates of the following prepared:

a) awns (after removal from the grain) (5/ml)

b) transparent layer of pericarp (10/ml)

c) green layer of pericarp (10/ml)

d) embryo (20/ml)

e) endosperm (including aleurone layer) (3/ml)

The fresh weights of each of the component tissues of the grain were determined by taking a sufficient number of tissues to weigh accurately, and weighing them immediately following dissection. Homogenates of fully expanded leaves of maize, pea, Sedum, barley (var. Julia) and wheat were also prepared.

II Assays

i) RBPC and PEPC

The activities of these enzymes were estimated as described in Chapter 2. For PEPC the buffered medium contained either 10 mM -cysteine or 10mM-mercoptoethanol. For RBPC the reaction medium contained 10 mM-cysteine and the reaction was initiated with the addition of 0.6 µmol RBP.

ii) GO

The method of Frigerio and Harbury[57] was modified to measure glycollate dependent oxygen uptake polarographically rather than manometrically. Homogenates of leaves of pea, maize, barley (Julia) wheat, Sedum and also pericarps of barley and wheat, were prepared in a similar medium to that used for the RBPC and PEPC assay except that it contained no $MgCl_2$ or $MnCl_2$.

A sample (2.7 ml) of homogenate was placed in the chamber of a Rank oxygen electrode, jacketed by a circulating water suppy at 30°C The cap was placed on the chamber, the solution allowed to warm and to reach a steady state of oxygen uptake as indicated on a chart recorder, flavin mononucleotide (0.3 ml 2mM solution) was added to the chamber and the rate of oxygen uptake recorded. The increase in rate following the addition of glycollate (0.03 ml M-sodium glycollate) was taken as a measure of glycollate oxidase activity. Results were expressed in μ moles of oxygen consumed per min per mg chlorophyll. Chlorophyll in samples of homogenate was estimated by the method of Arnon[6].

iii) Chlorophyll a : Chlorophyll b Ratios.

Two methods were used to determine the chlorophyll a : b ratios.

(a) Method of Arnon[6]

Sufficient acetone was added to tissue homogenates (prepared as for PEPC assay) to bring the concentration to 80% acetone (v/v). The absorption co-efficients of chlorophylls at 645 and 663 nm were used to calculate the chlorophyll a : b ratios, i.e.

$$\text{Chlorophyll a(mg/l)} = 12.7\ A_{663} - 2.9A_{645}$$

and

$$\text{Chlorophyll b(mg/l)} = 22.9A_{645} - 4.68A_{663}$$

where A_{645} and A_{663} are the absorbances at 645 and 663 nm respectively.

(b) Method of Wintermans and Demots[146]

Tissue was homogenised in 96% ethenol at 70°C and the absorption coefficients of chlorophylls at 649 nm and 665 nm were used to calculate the chlorophyll a : b ratios i.e.

Chlorophyll a (mg/l) = $13.70\ A_{665} - 5.76\ A_{649}$

and

Chlorophyll b (mg/l) = $25.80\ A_{649} - 7.60\ A_{665}$

where A_{649} and A_{665} are the absorbances at 649 nm and 665 nm respectively.

iv) PEP synthetase

The reaction medium was that used for PEPC estimation. Pyruvate dependent carbon dioxide fixation was initiated by adding substrate to the reaction medium containing respectively:

(a) 5mM-pyruvate

(b) 1.5 mM-ATP

(c) 5mM pyruvate and 1.5 mM-ATP

(d) 5mM-disodium orthophosphate and 1.5 mM-ATP

(e) 5mM-disodium orthophosphate, 5mM-pyruvate and 1.5 mM-ATP

The results were compared with samples to which no substrate had been added i.e. containing no pyruvate, ATP or orthophosphate.

v) PEP Carboxykinase

PEP carboxykinase activity was measured by a method similar to that described by Edwards et al.[45] in which the reaction was followed in the direction of OAA synthesis and carbon dioxide uptake.

Results

I RBPC and PEPC

The activities of RBPC and PEPC in a number of plant tissues are shown in Table 3.1. On a chlorophyll basis the leaves of maize (C_4)

Table 3.1 Activities of RBPC and PEPC in Various Plant Tissues at 30°
(Activity expressed in $\mu mol/CO_2/min/mg$ Chlorophyll.

Plant Tissue	RBPC Medium Containing 10mM-cystine	PEPC Medium Containing 10mM-cysteine	PEPC Medium Containing 10 mM - Mercaptoethanol	RBPC/PEPC Activity Ratio
Maize leaf	7.1	16.90	2.8 ± 1.3	0.42
Pea Leaf	8.5	0.05	-	170
Barley Leaf	1.4	0.05	0.3 ± 0.1	28
Barley Awn	2.4	0.27	-	0.89
Barley Pericarp	0.46	1.10	1.8 ± 0.5	0.42
Sedum Leaf	-	-	0.64 ± 0.01	-
	Figures are averages of duplicate estimates		Figures averages of four estimates $\pm$ standard deviation.	

and pea (C_3) had similar activities of RBPC. Activities were greater than those found in each of the barley tissues. For barley, the RBPC activity was greatest in the awn and least in the pericarp, the leaf had an intermediate level.

For the estimation of PEPC either cysteine or mercaptoethanol was included in the reaction medium. The PEPC activity measured in maize leaf, in a medium containing 10mM-cysteine was greater than that in 10mM-mercaptoethanol, for the barley pericarp the opposite effect was observed. However, for both methods of determining PEPC activity, the greatest activity was found in maize leaves, and least in barley leaves, the barley pericarp had an intermediate level of PEPC activity. Sedum (CAM) PEPC activity was greater than that of the barley leaf yet less than that found in the barley pericarp.

Also shown in Table 3.1 are the ratios of RBPC to PEPC (both estimated in a medium containing 10mM-cysteine) The RBPC/PEPC activity ratio was highest for pea leaf. The ratio of activities for maize leaf and barley pericarps were identical, this was also the lowest value found in the range of tissues studied.

II Glycollate Oxidase

The activities of GO from various plant tissues are shown in Table 3.2. The highest level of enzyme activity was recorded in the wheat leaf, followed by that of Sedum and pea leaves. These values were approximately twice those found in the leaves of maize and barley. The pericarps of wheat and barley had significantly less GO activity than the leaves of the respective plants.

III Chlorophyll a : Chlorophyll b Ratio.

The Chlorophyll a : Chlorophyll b ratios for various plant tissues are shown in Table 3.3. Arnon's method[6] following acetone extraction gave chlorophyll a : b ratios which varied very little throughout the range

Table 3.2

Levels of Glycollate Oxidase in Plant Tissues

/umoles oxygen/min/mg chlorophyll

Plant Tissues	Glycollate Oxidase
Pea Leaf	1.05 ± .11
Sedum Leaf	1.12 ± .47
Maize Leaf	0.50 ± .12
Julia Leaf	0.60 ± .12
" Pericarp	0.16 ± 0.08
Maris Dove Leaf	1.38 ± 0.02
" " Pericarp	0.65 ± 0.04

Figures averages of three estimates ± standard deviation.

Table 3.3 Chlorophyll a : Chlorophyll b ratios

Tissue	Chlorophyll a/b method of Arnon	No. of Readings	Chlorophyll a/b method of Wintermans and Demots	No. of Readings	A/W	Chlorophyll a Extracted by Hot Alcohol %
Pea Leaf	1.93±0.15	4	1.96±0.17	10	0.99	1
Maize Leaf	1.84±0.11	6	2.85±0.24	7	0.65	35
Barley Leaf (Var. Julia)	1.41±0.25	6	1.67±0.13	4	0.85	15
Pericarp (Var. Julia)	1.54±0.43	14	2.18±0.29	8	0.71	29
Pericarp (Var. Maris Dove)	-	-	1.84±0.29	10	-	-
Awn (Var. Julia)	2.04±0.43	5	-	-	-	-
Sedum Leaf	1.61± 0.20	4	2.09±0.32	5	0.78	22

of tissues studied. The mean chlorophyll a : b ratio was 1.76 and all estimates include this value in their range. The average chlorophyll a : b ratio using Wintermans and Demots' method[146] following hot alcohol extraction of the various tissues was 2.10, a value which was neither in the range for maize leaves (which had a significantly higher value) nor in that of barley leaves (which was significantly below average value). Also shown in Table 3.3 is the result of dividing the chlorophyll a : b ratio determined by Arnon's method (A) by the ratio determined by Wintermans and Demots' (W). This quotient (A/W) is in all cases less than one, indiciating that the estimate of chlorophyll a was greater (or chlorophyll b less) when estimated by Wintermans and Demots' method. The quotient was least for the maize leaf and highest for the pea leaf. If it is to be assumed that the only difference in the two methods is that hot alcohol extracts a quantity of the total chlorophyll a which is not extracted by 80% acetone, then

$$A = \frac{a}{b}$$

where a = the amount of chlorophyll a extracted in 80% acetone

b = the amount of chlorophyll b extracted by both 80% acetone and hot ethanol

and $$W = \frac{a + x}{b}$$

where x = the further amount of chlorophyll a extracted by hot ethanol

Thus, the fraction of total chlorophyll a extracted only by the hot alcohol treatment eqals $\frac{x}{a + x}$ = $(1 - \frac{A}{W})$ or expressed as a percentage = $100(1 - \frac{A}{W})\%$

The amount of chlorophyll a extracted by hot alcohol in excess of extraction in 80% acetone (x) is shown in the right hand column of Table 3.3. This value is highest for maize leaves and lowest for

pea leaves. The value of 'x' for barley pericarps was between that for leaves of maize and Sedum.

IV PEP Synthetase

The rates of CO_2 fixation by homogenates of maize leaves and barley pericarps after addition of the various substrates are shown in Table 3.4. Maximal rates of $C^{14}O_2$ fixation in maize leaves were obtained only when all three substrates (pyruvate, ATP and orthophosphate) were present. For barley pericarps, the highest rates of $C^{14}O_2$ fixation were observed when ATP alone was added. The further addition of pyruvate, and pyruvate and orthophosphate, decreased the rate of $C^{14}O_2$ fixation of this tissue.

V PEP Carboxykinase

The activity of this enzyme in the barley pericarp was 30 ± 6 nmol CO_2/min/mg chlorophyll and in maize leaf 73 ± 18 nmol CO_2/min/mg chlorophyll. These were the means of four estimates.

VI Pericarp PEPC Activity

The activity of PEPC in the pericarps of various cereals are given in Table 3.5. There was a wide range of activity, the highest total activity being found in oats, wheat and barley (var. Sentor) were not significantly less. On a chlorophyll basis, the highest activity was found in wheat. The total PEPC activity of barley (var. Julia) was near the average (9.88 nmol CO_2/min/pericarp) The lowest activity was observed with the mutant barley Albino Lemma. On both a chlorophyll basis and a per tissue basis the Scottish Bere barley had a very low activity.

VII PEPC Activity in Different Grain Tissues

The activities in this enzyme in the tissues of the immature grain of barley var. Julia are shown in Table 3.6. The greatest activity

Table 3.4 PEP Synthetase Activity of Maize Leaves and Barley Pericarps

Addition to Homogenates	Rate of $C^{14}O_2$ Fixation	
	Maize Leaf	Barley Pericarp
Pyruvate (5mM)	42.9 ± 3.9	25.6 ± 2.3
ATP (1.5mM)	27.6 ± 2.6	250.0 ± 32.0
ATP (1.5mM) + Pyruvate(5mM)	55.2 ± 6.1	229.0 ± 26.0
ATP (1.5mM) + Pyruvate(5mM) + Orthophosphate (5mM)	238.0 ± 60.0	165.0 ± 17.0

Figures are averages of four readings ± standard deviations
Measured in nmol CO_2/min/mg Chlorophyll

Table 3.5
The Activity of PEPC in Cereal Pericarps

Pericarp Source	Total Activity nmol/CO_2/min/ Pericarp	Activity/mg Chlorophyll µmol/CO_2/ min/mg Chlorophyll
Barley (var.Julia)	6.64 ± 0.37	1.80 ± 0.50
Barley (var.Sentor)	15.93 ± 0.95	2.85 ± 0.17
Barley (var. Scottish Bere)	1.55 ± 0.02	0.29 ± 0.01
Barley (var.Albino Lemma)	1.43 ± 0.05	-
Wheat (var.Maris Dove)	16.10 ± 1.89	11.10 ± 1.30
Oats (var.Astor)	17.60 ± 0.90	7.5 ± 0.38

Figures are averages of four readings ± standard deviations

Table 3.6
Activity of PEPC in Component Tissues of the Grain of Barley (var. Julia) 25 days after Anthesis

Tissue	Total Activity nmol CO_2/min/tissue	Relative Activity mmol CO_2/min/ g fresh weight
Embryo	1.09 ± 0.14	1.25 ± 0.29
Transparent layer of Pericarp	1.30 ± 0.14	1.00 ± 0.29
Green layer of Pericarp	6.64 ± 0.37	1.70 ± 0.52
Endosperm (+Aleurone Layer)	9.00 ± 2.00	0.17 ± 0.05

Figures are averages of four readings ± standard deviation

appears in the endosperm. This activity, however, was not significantly greater than that found in the green layer of the pericarp. Comparison on a fresh weight basis showed the relative activity to be greatest in the green layer of the pericarp, but it was also high in both embryo and transparent layer of the pericarp.

Discussion

I RBPC

The activity of RBPC on a per mg chlorophyll basis was similar to that reported by Björkman and Gaul[20]. In the present work only small amounts of leaf tissue were used, and thus complete homogenisation was easily effected. It would thus appear that the lower activity in C_4 plant leaves reported by Slack and Hatch[121] was indeed due to incomplete homogenisation of the tissue. Therefore it is not possible to take the RBPC activity per mg chlorophyll as an indication of the pathway of CO_2 fixation in a particular tissue.

II PEPC

The activities of PEPC (expressed in μmol CO_2/min/mg chlorophyll) estimated in 10mM-cysteine are similar to those reported by Slack and Hatch[121] i.e. they are high for C_4 plant leaves and low in C_3 leaves. It is therefore possible to use the activity of this enzyme as an indication of the pathway of CO_2 fixation in a particular tissue. However, the activity of this enzyme in barley pericarp tissue was between those of 'typical' C_4 and C_3 plants. The estimate of PEPC activity carried out in 10 mM-mercaptoethanol also gave a result difficult to classify.

III Ratio of RBPC : PEPC Activities.

Comparisons made on a chlorophyll basis (see above) have limitations, in particular it is known that the chloroplasts of plants adapted to shady natural habitats contain more chlorophyll than those of plants

from sunny habitats[3]. It might be expected therefore that a similar situation would exist for the pericarp, shaded as it is by other tissues of the ear. A high chlorophyll content would lead to low levels of PEPC and RBPC when expressed on a chlorophyll basis. To overcome these difficulties comparisons were attempted using the RBPC/PEPC activity ratio for each tissue. Using the data of Slack and Hatch[121] this ratio was a value of 14.0 ± 1.5 in C_3 plants and 0.025 ± 0.008 in C_4 plants, or using the data of Björkman and Gaul[20], the RBPC/PEPC activity ratio has a value of 7.25 ± 2.25 in C_3 plants and 0.31 ± 0.08 in C_4 plants.

The ratios estimated in the present work are given in Table 3.1 and RBPC/PEPC activity ratios were identical (0.42) for maize leaf and barley pericarp, which may indicate that these tissues have similar pathways of CO_2 fixation. The barley awn also had a low value for this ratio which indicates that this tissue may have unusual properties. The products of $C^{14}O_2$ fixation in awns have been determined and no trace of the characteristic C_4 intermediates was found (C.M. Willmer personal communcation).

IV Level of GO Activity.

Homogenisation techniques used for the various tissues were adequate enough to confirm the high level of RBPC in maize, previously reported by Björkman and Gaul[20], and therefore the low level of GO activity found here is unlikely to be a result of incomplete homogenisation of the maize leaf. Thus the present work is in agreement with the previous work of Tolbert et al.[136] i.e. that C_4 plants have a low GO activity per mg chlorophyll.

The barley leaf, however, did not have a significantly greater activity of GO than the maize leaf. It had a low RBPC activity (on a chlorophyll basis). Subject to the limitations of comparison of enzyme

activities on a chlorophyll basis, these results indicate that enzyme activity is generally low in barley leaves. This low activity may account for the low relative contribution of the leaves to dry matter in the grain of barley[4, 56, 115]. The level of GO activity (on a per mg chlorophyll basis) found in the pericarps of wheat and barley was lower than that for the respective leaves, indicating that the pericarps have a lower capacity for photorespiration than the leaves.

V Chlorophyll a : Chlorophyll b Ratios.

All tissues gave the same ratio of chlorophyll a : b when extracted in 80% acetone and Arnon's[6] method of chlorophyll determination used. The solids filtered from homogenates in 80% acetone were green, indicating that chlorophyll extraction by the method of Arnon[6] was not complete. Following extraction in hot alcohol however any solids were devoid of colour. The difference between the two sets of results could therefore be explained by the difference in efficiency of extraction. The chlorophyll a : b ratios were higher in hot ethanol extracts of tissues, thus indicating either that the fraction not extracted by 80% acetone is principally chlorophyll a or that chlorophyll b is degraded by the heat treatment. While the method of tissue homogenisation appeared adequate for extraction of water soluble enzymes, the hydrophobic nature of chlorophyll may explain the difficulty in complete extraction of chlorophyll a.

With pea leaves on the other hand, 99% of the chlorophyll a was extracted in 80% acetone. The opposite was the case for the maize leaf. The retention of chlorophyll a may be a property of C_4 plants. The quantity of chlorophyll a retained by pericarps was between that of Sedum and maize leaves. This again may indicate that the pericarp properties are intermediate between C_4 and CAM plants.

VI Enzymes of PEP formation

Since PEP carboxykinase is located in the bundle sheath of only a few C_4 plants[68], it is not generally accepted as the enzyme which catalyses the regeneration of the CO_2 acceptor, PEP. The activity of PEP carboxykinase estimated here in maize leaves is one hundred times less than the activity of RBPC (Table 3.1). Thus, if PEP carboxykinase was responsible for PEP regeneration, it would become the rate limiting step of photosynthesis. This is unlike the situation in Panicum maximum[70], in which the enzymes RBPC and PEP carboxykinase have similar activity. The activity of PEP carboxykinase was only 7% of that of RBPC in the barley pericarp. Hence, if the pericarp does have C_4 metabolism and if PEP carboxykinase is the major enzyme of PEP regeneration, the formation of PEP will be the rate limiting step of CO_2 fixation. The activity of PEP synthetase was greater than the activity of PEP carboxykinase in both maize leaves and the barley pericarp. However, it is difficult to ascribe the CO_2 fixation recorded to the PEP sythetase reaction, as the addition of ATP to homogenates of pericarp tissue caused even higher rates of CO_2 fixation than the addition of all three substrates of PEP synthetase (ATP, pyruvate and orthophosphate). It is not clear whether the ATP dependent CO_2 fixation in barley pericarp is due to PEP synthesis occurring in the presence of endogenous pyruvate and orthophosphate or whether it is due to some other enzyme e.g. ribulose 5 - phosphate kinase which catalyses the synthesis of RBP. There may well be a novel pathway for the synthesis of PEP in the barley pericarp. It is of course possible that the techniques employed here for the measurement of PEP synthetase have resulted in the enzyme showing less than its in vivo activity, for example the enzyme may be particularly sensitive to inactivation by quinones (See Chapter 2).

VII PEPC Activity of Cereal Pericarps.

In general the cereal pericarps contained a high level of PEPC, exceptions were the pericarps of the barleys var. Scottish Bere and var. Albino Lemma. It is possible that the activity of PEPC in the pericarp is proportional to the photosynthetic activity of the tissue, as is known to be the case with greening sugar cane leaves[59]. Albino Lemma had the lowest level of PEPC activity and this may be correlated with the fact that this tissue is non-photosynthetic. Scottish Bere also had a low activity of PEPC. This variety has been superceded by higher yielding varieties of barley (R.C.F. Macer personal communication). It is possible that the low yield of Scottish Bere is a result of the low PEPC activity of the pericarp.

It is observed in the course of these experiments that the maturation of the oat grain was different from that of wheat and barley. In oats, the apex of the grain matured a long time before the base, while in wheat and barley the maturation was not polarised to the same extent. This different development pattern made comparison with wheat and barley difficult, therefore it was considered impracticable to include oats in further studies.

VIII PEPC Activity in Different Grain Tissues.

It has been shown here that the non-photosynthetic pericarp of Albino Lemma (Table 3.5) has measurable PEPC activity, as did component tissues of the grain (Table 3.6). It is possible that the enzymes in these tissues as in micro organisms[83] and the leaves of C_3 plants[121] 'top up' the levels of Krebs cycle intermediates. This anaplerotic function of PEPC is probably most important in cells synthesising protein since a number of Krebs cycle intermediates can be transaminated to form amino acids. Mature barley grain contains 7.5 - 10% protein[93], thus significant protein synthesis takes place.

PEPC may have a function in the synthesis of protein. On the other hand the enzyme may be located throughout the grain to trap CO_2. The products of this reaction may be transported to the pericarp (or other photosynthetic tissues) for reduction. The function of grain PEPC could only be discovered by following the products of CO_2 fixation in each of the tissues.

Conclusions

The pathway of CO_2 fixation can only be determined by studying the products of photosynthesis. However an indication of the possible metabolic events may be gained from studying the enzyme complement of pericarps, as suggested by Chang and Troughton[30]. The activity of each of the enzymes studied in the pericarp, was not identical to those estimated in plants typical of each group of carbon fixation i.e. C_3 C_4 and CAM. Table 3.7 shows each of the tests which may differentiate the classes of carbon dioxide fixation. Asterisks show the pathways of carbon fixation of barley pericarps which are consistent with each test. From this table it is clear that the pericarps of barley share many properties with the leaves of C_4 plants. The problem of PEP regeneration in the barley pericarp is however still unsolved since the presence of PEP synthetase in this tissue was not demonstrated satisfactorily.

Nevertheless, the results indicate the presence of novel characteristics in the pericarp and that the mechanism of CO_2 fixation may be a novel one. The low level of PEPC activity found in the chlorophyll-less pericarps of Albino Lemma , and the high activity found in green pericarps indicate that PEPC has some function in photosynthesis.

Table 3.7
Pathways of CO_2 fixation Inferred by a Series of Assays

Test	Inferred Pathway (indicated by asterisks)		
	C_3	CAM	C_4
Level of PEPC Activity		✪	✪
RBPC/PEPC ratio			✪
Level of GO Activity	✪		✪
Chlorophyll a : b ratio		✪	✪

Chapter 4

Oxygen Exchange in Detached Grains of Barley and Wheat

Introduction

It was noted previously (Chapter 3) that the enzyme levels in the barley pericarp (var. Julia) on a chlorophyll basis were low compared to enzyme levels in leaves of C_4 plants. This was true not only for PEP carboxykinase, PPO (Chapter 2), GO and RBPC, but also for PEPC. These low levels may be artificially low due to the possibly inappropriate choice of chlorophyll as a basis for comparison i.e. chlorophyll levels may be unusually high due to the shaded environment of this tissue. On the other hand they may simply reflect a genuinely low metabolic activity.

Oxygen evolution was therefore measured in an attempt to assess the ability of the pericarp to carry out photosynthesis.

Methods

The barleys (var. Julia and Albino Lemma) and the wheat (var. Maris Dove) were grown as described in Chapter 2.

In the case of the barleys, grain were removed at 25 days after anthesis. For wheat, spikelets and grain were removed at the stage of development most closely corresponding to that of the barleys at 25 days. Spikelets of wheat (containing two or three fertile grain) were removed from the rachis intact, this was not possible for the barleys as the individual grains of the spikelets are attached directly to the rachis. For the measurement of O_2 evolution, two spikelets of wheat or three grains were placed in the chamber of an oxygen electrode containing 3.0 ml of buffer (0.33 M-sorbitol, 50mM-tricine KOH pH 7.5 1 mM-$MgCl_2$ and 1 mM-$MnCl_2$) If more than three grains were used the rate of oxygen evolution was found to be sufficient to saturate the liquid in the chamber. The oxygen electrode was jacketed by a circulating water

supply at 30°C. An aluminium foil shade was placed over the electrode to exclude as much light as possible. The rate of oxygen evolution was recorded on a chart recorder. The shade was then removed and the chamber illuminated with a 270 W incandescent electric lamp (Philips No. 1 Photoflood), the bulb of which was a distance of 150mm from the centre of the chamber. After allowing time for the system to reach a new steady state, the new rate of oxygen uptake was recorded. The rate of oxygen evolution in the dark was subtracted from that in the light to give the rate of light dependent oxygen evolution. The rate of respiration was assumed to be constant in the dark and in the light. This is certainly true for all the non photosynthetic tissues of the grain. The presence of photorespiration in green tissue would result in an increased oxygen uptake in the light compared to that in the dark, thus light dependent oxygen evolution would be underestimated. Since the pericarp has some of the character of the C_4 leaf (high PEPC activity, low GO activity (Chapter 3)), it would be expected that this tissue would have a low rate of photo-respiration. The experiment was repeated three times and the mean of each of the three rates calculated. The rate of oxygen production was expressed in nmoles O_2/min/grain. The three rates as described above were also measured for wheat grain after successive removal of the husk (palea and lemma), the transparent layer of the pericarp, and the green layer of the pericarp. In the case of the barleys oxygen evolution was measured with the awn removed (because the grain with awn attached was too large to fit in the chamber), and then after successive removal of the different layers as described for wheat. Since Albino Lemma does not have a green pericarp, the final stage was necessarily omitted. Light dependent oxygen evolution was also measured in the isolated husks and in isolated barley pericarp, 0.3 ml of 0.5 mM-sodium bicarbonate

was added to the suspension of the isolated pericarps and the differences in oxygen evolution in the light noted.

Results

The rate of oxygen uptake in the dark was considered to be an estimation of respiration, while the rate of light dependent oxygen evolution was considered to be an estimate of the rate of photosynthesis. The rates of oxygen uptake in the light and in the dark, together with the light dependent oxygen evolution of the grain and grain fractions of Albino Lemma are shown in Table 4.1. There was no net oxygen evolution in the light in the intact grain, that is, light dependent oxygen evolution did not exceed the rate of oxygen uptake. Removing the husk and the transparent layer increased the rate of measured O_2 evolution of the remaining tissues but succeeding results were not significantly different.

The corresponding rates for the grain of wheat are shown in Table 4.2. Oxygen evolution in the light was observed with the floret, the whole grain, the grain with husk removed, and the transparent layer of the pericarp removed. The isolated husk showed a high rate of light dependent O_2 evolution (6.3 nmol O_2/min/grain) compared to that for the whole grain (10.0 nmol O_2/min/grain). However, removal of the husk caused an increase in light dependent oxygen evolution to 18 nmol O_2/min/grain, a larger increase than that measured after removal of the transparent layer of the pericarp (21 nmol O_2/min/grain). It was only when the green pericarp was removed that the rate of oxygen evolution fell from a rate of +5 nmol O_2/min/grain to -11 nmol O_2/min/grain. There was little difference in this case between results in light and dark.

The results for similar experiments with barley var. Julia are shown in Table 4.3. The whole grain, or with the husk, or husk and

Table 4.1

Oxygen Exchange in Barley (var. Albino Lemma)

Tissue	Oxygen Exchange		
	Light	Dark	Light Dependant O_2 Exchange
Grain (awns only removed)	-0.9 ± 0.4	-3.4 ± 0.8	$+2.9 \pm 0.3$
Husk removed	-6.3 ± 0.6	-7.2 ± 0.3	$+1.0 \pm 0.6$
Transparent layer of Pericarp removed	-6.6 ± 0.4	-8.4 ± 0.4	$+1.8 \pm 0.6$
Isolated Husk	-1.1 ± 0.1	-3.4 ± 0.3	$+2.3 \pm 0.3$

Figures are averages of three estimates $\pm$ standard deviation expressed in nmol/O_2/min/grain or tissue fraction

+.....O_2 evolution

-.....O_2 uptake

Table 4.2
Oxygen Exchange in Wheat (var. Maris Dove)

Tissue	Oxygen Exchange		
	Light	Dark	Light Dependent O_2 Evolution
Floret	2.0 ± 0.04	-9.0 ± 2.0	$+11.0 \pm 2.0$
Grain	$+3.0 \pm 0.3$	-7.0 ± 0.00	$+10.0 \pm 0.3$
Husk Removed	$+9.0 \pm 2.0$	-9.0 ± 0.3	$+18.0 \pm 1.0$
Transparent Layer of Pericarp Removed	$+5.0 \pm 3.0$	-16.0 ± 2.0	$+21.0 \pm 4.0$
Green Layer of Pericarp Removed	-11.0 ± 1.0	-13.3 ± 1.0	$+ 3.0 \pm 2.0$
Isolated Husk	$+2.0 \pm 1.0$	$- 4.0 \pm 0.3$	$+ 6.3 \pm 1.0$

Figures are average of three estimates $\pm$ standard deviation expressed in nmol O_2/min/grain or tissue fraction.
+ ... O_2 evolution
- ... O_2 uptake

Table 4.3
Oxygen Exchange in Barley (var. Julia)

Tissue	Oxygen Exchange		
	Light	Dark	Light-Dependent O_2 Evolution
Grain (awns only removed)	+5.4 ± 1.6	-3.8 ± 0.8	+9.4 ± 1.1
Husk Removed	+4.3 ± 2.9	-8.2 ± 0.6	+12.5 ± 3.4
Transparent Layer of Pericarp Removed	±1.2 ± 3.5	-13.3 ± 0.6	+17.3 ± 1.7
Green Layer of Pericarp removed	-8.4 ± 0.2	-10.3 ± 0.5	+1.8 ± 0.2
Isolated Husk	-1.0 ± 0.4	-6.2 ± 1.4	+5.3 ± 1.8
Isolated Green Layer of Pericarp	-2.3 ± 0.3	-2.8 ± 0.2	+0.5 ± 0.0

Figures are averages of three estimates ± standard deviation expressed in nmol O_2 /min/grain or tissue fraction
+ .....O_2 evolution
- .....O_2 uptake

transparent layer of the pericarp removed, evolved oxygen in the light. The removal in turn of husk and transparent layer resulted in a sharp increase in dark O_2 uptake. Removal of the green layer of the pericarp caused a decrease in dark O_2 uptake and a dramatic fall in light dependent O_2 evolution. With the isolated husk, light dependent O_2 evolution was 5.3 nmol O_2/min/grain, compared with 0.5 nmol O_2/min/grain for the isolated green layer of the pericarp. The rate of oxygen uptake of isolated green pericarps was 3.3 $\pm$ 0.4 nmoles O_2/min/pericarp in the light, with 50 mM-sodium bicarbonate added to the medium it was 2.4 $\pm$ 4 nmol O_2/min/pericarp, indicating a net rate of light dependent oxygen evolution of +0.8 $\pm$ 0.4 nmol O_2/min/pericarp.

Discussion

It has been observed that the inner surface of the palea and lemma of wheat have no stomata[128]. If these tissues are firmly attached, which they appear to be at the age investigated here, then it is likely that they form a gas tight enclosure round the grain. The transparent and green layers of the pericarp may also form similarly impervious layers[49]. Thus the results reported here must relate only to those tissues outside any such gas tight layers. For example, the rate of oxygen uptake in the dark by intact grains of Albino Lemma was 3.4 $\pm$ 0.8 nmoles O_2/min/grain. This figure is probably not that for the intact grain but only for those tissues outside the gas tight layer. Removal of the palea and lemma caused the light and dark rates of respiration to increase. If the outer tissues were completely permeable to gases, the respiration rate would be expected to fall on their removal, as these tissues would no longer be present to add their oxygen consumption to the total. It does appear therefore that the palea and lemma do form a layer resistant to gaseous diffusion around the grain. The rates of oxygen uptake of the intact grain are

almost identical to those of the isolated husk, suggesting that the rates of oxygen production measured for the intact grain were accounted for by this tissue. As respiration exceeds photosynthesis in the husk, photosynthesis by lemma and palea of Albino Lemma barley can then make no contribution to grain filling.

The rate of respiration of Albino Lemma observed with the husk removed may not represent the in vivo rate. If as suggested the husk forms a seal round the grain, and prevents entry of oxygen to the inner tissues, then the in vivo rate of respiration may therefore depend on the availability of oxygen. Removal of the husk would increase oxygen availability and hence the measured respiration rate. If the husk prevents both the inward flow of oxygen and the outward flow of carbon dioxide, carbon dioxide concentration may increase within the inner tissues until respiration is limited by oxygen availability.

The small increase in dark respiration caused by removal of the transparent layer of the pericarp may indicate either that this tissue also is not freely permeable to gases or that it is completely permeable and its removal results in no change at all. However, as the rates of respiration in the light are not significantly different with or without the transparent layer of the pericarp, the situation regarding the permeability of this layer is not so clear as for the husk. It has been shown[118] that the ability of the green layer of the pericarp to fix externally supplied $C^{14}O_2$ is much increased by removal of the transparent layer, a result which indicates strongly that the transparent layer is indeed impervious to gases.

The dark respiration of the whole grain of barley var. Julia is similar to that of Albino Lemma, but the light dependent O_2 evolution by intact barley var. Julia grain was over three times that of Albino Lemma. Since the only difference between the two is the green peri-

carp layer of barley var. Julia, this must be due to the O_2 evolution by this layer. As with Albino Lemma, the isolated husks of Julia had a net O_2 uptake in the light therefore photosynthesis by the husk probably made no contribution to grain filling. The higher rate of dark respiration of the isolated husk of Julia compared with the intact grain may have been due to the greater exposed surface area of the husk when detached from the grain, as the inner surfaces were then exposed to the medium. Prior to isolation, the only route of O_2 entry was through the stomata of which there were few[105] or the cut tissue where the awn had been removed. The inner surfaces of the husk of barley var. Julia may therefore be more permeable to oxygen than those of Albino Lemma. However, these results may be explained by a greater availability of respiratory substrate in the husk of barley var. Julia.

Removal of the husk from the grain of Julia not only increases the rate of apparent respiration of the grain but also the rate of light dependent oxygen evolution, although the large error in the last figure (12.5 ± 3.4 nmol O_2/min/grain) makes the difference between the whole grain and the grain without husk not significant. However, the trend continues with the removal of the transparent layer of the pericarp, both respiration rate and light dependant oxygen production being greater than for the intact grain. Thus, the husk and transparent layers may also prevent diffusion of oxygen from the grain as well as preventing its entry into the grain.

Since Evans and Rawson[49] found that the husk filters 78% of the light, the increased rate of light dependent oxygen evolution after removal of the husk and transparent layer of the pericarp may be due to the increased light falling on the pericarp. These workers[49] found that photosynthesis of wheat pericarps (as measured by CO_2 uptake using infra red gas analysis) is saturated at very low light intensities.

The present work and that of Radley[118] indicates that the transparent layer, like that of wheat, may not be permeable to gases. Thus, if the carbon dioxide fixed by the pericarp originates from the atmosphere, the early saturation kinetics of photosynthesis may be due to the transparent layer preventing entry of carbon dioxide and efflux of oxygen. This applies only if carbon dioxide originates from the atmosphere.

The results of the experiments reported here with wheat agree with those for barley. The arrangement of the floret of wheat seems to confer no significant advantage in terms of grain photosynthesis.

Additionally it is clear that the green layer of the pericarp is capable of high rates of oxygen evolution while attached to the grain. If it is assumed, 1) that the rate of light dependent oxygen evolution, for example that measured for the grain of Julia with the husk only removed, is the *in vivo* rate of photosynthesis, 2) that this rate is maintained over the eighteen 'light' hours for each of the thirty days that the pericarp is functional, and 3) that the photosynthetic quotient is unity, then the total amount of CO_2 fixed by the pericarp can be estimated. This was found to be 12.0 ± 3.3 mg The average weight of a grain of Julia at maturity is 40mg[145]. Thus, a large proportion (30%) of the carbohydrate of the grain may have been formed from carbon dioxide fixed by the pericarp. The relative contribution of photosynthesis by the ear to grain filling was assessed by ear shading to be about 30%[18,133]. Thus it is possible that the pericarp makes the major, if not the sole contribution to net ear photosynthesis.

Isolated pericarps of Julia were not capable of the high rates of oxygen production observed when attached to the grain. This may have been due to the absence of carbon dioxide, which is probably supplied at a high rate by respiration of the embryo and endosperm when the

pericarp is attached to the grain. The addition of bicarbonate increased oxygen production, but this rate was only 5% of that produced when the pericarp was attached to the grain. Baxter[13] was able to show light dependent oxygen production by isolated pericarps of Maris Baldric barley using dichlorophenolindophenol as an electron acceptor, however this does not establish that carbon dioxide is the final electron acceptor. In the present work studies were carried out in an isotonic medium in order to minimise the outward diffusion of photosynthetic intermediates from the cells of the pericarp which would result in reduction of the rate of photosynthesis. It is possible, however, in spite of these precautions, that some such outward diffusion took place, thus causing an apparently low rate of photosynthesis. In vivo this ease of outward diffusion from the inner surface of the pericarp may assist exchange of metabolites between the pericarp and aleurone layer.

Conclusions

The husk and transparent layer of the pericarp may prevent the escape of respired CO_2 from the grain and the resulting increase in internal CO_2 levels should promote grain photosynthesis, and calculations suggest that the green layer is certainly capable of high rates of oxygen evolution in the light. It is therefore likely that the apparently low levels of pericarp enzyme activity reported in Chapter 3 are not due to a generally low metabolic activity.

It may also be concluded, since it appears that the husk and transparent layer reduce the general diffusion of gases into and from the inner tissues of the grain, that the rate of endosperm and embryo respiration may be limited by the supply of atmospheric oxygen. Thus, the rate of respiration may depend on the rate of oxygen production by the pericarp. The pericarp therefore may function not only as a

translocating tissue supplying the endosperm[55], and as a photosynthetic organ, but also as a means for controlling the metabolic activity of the endosperm.

Chapter 5

Changes in Activity of PEPC during Grain Development

Introduction

It was noted in Chapter 3 that the activity of PEPC in chlorophyll containing pericarps was greater than that observed in the chlorophyll lacking pericarps of Albino Lemma barley. It was suggested that PEPC may be involved in photosynthesis by the green pericarps. Duffus and Rosie[41] reported that the chlorophyll content of barley (var. Julia) pericarps changed during development, increasing rapidly in the first twenty days of development and reaching a maximum at about twenty seven days after anthesis. Thereafter the chlorophyll content of the pericarp declined reaching zero at about fifty five days after anthesis. It was therefore of interest to follow the changes of PEPC activity and to correlate activity of this enzyme with the chlorophyll content of the pericarp.

Methods

The date of anthesis was determined for various samples of barley (Hordeum distichum var. Julia) by the method of Merritt and Walker[103]. Ears were tagged at anthesis and collected the required number of days later. This method of defining the stage of development of the grain has severe limitations as the rate of development of the grain is assumed to be independent of growth conditions. Thus, it is not possible to study the effects of growth conditions on grain development using this method.

Baxter[13] outlined a series of morphological changes which occur during the development of Maris Baldric barley, and used them as a standard description of age of developing grain. Grain from the 1974, 1975, and 1976 seasons and thus subject to different environmental conditions was grown on the farms of the School of Agriculture, University of

Edinburgh and harvested at intervals throughout development. Morphological age was estimated according to the scheme of Baxter[13] (Table 5.1).

Variation in PEPC activity and chlorophyll content with time was determined from the results for barley grown (as described in Chapter 2) during March and April (1976) and July and August (1975). Similarly a time course of RBPC activity was prepared for barley earing in March and April (1976). These results were compared with the PEPC and chlorophyll time courses from the pericarps of barley grown from seed in an illuminated incubator (20 h day, total of 7,000 lux supplied with a combination of fluorescent and incandescent lamps) and maintained at either 10°C or 25°C.

Methods of enzyme assay and chlorophyll estimation have been described in Chapter 3.

Results

I Morphological Development of Grain.

Fig. 5.1 shows the stages of morphological development assessed by the method of Baxter[13] for grain produced in 1974, 1975, and 1976. The solid line is that for Maris Baldric and represents the relationship between chronoligical age and morphological age as described by Baxter. The results obtained here can thus be compared using Maris Baldric as a reference (with a gradient of unity and passing through the origin). Points falling below this line indicate a development slower than that of Maris Baldric, points above this line (i.e. a slope greater than unity) indicate a faster rate of development.

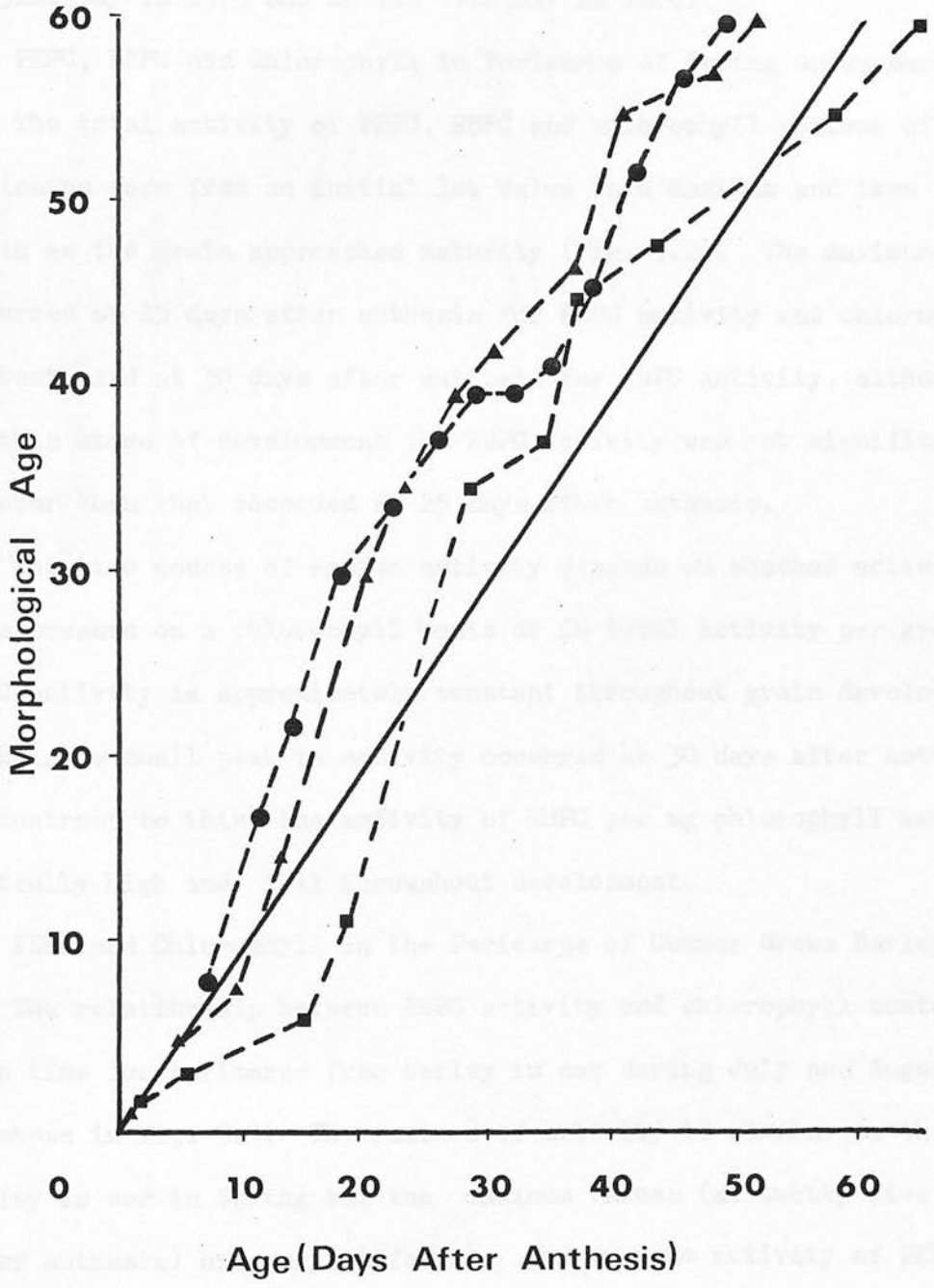
The development of the 1974 crop was slow in the first 18 days after anthesis, but by 28 days was in advance of that predicted for Maris Baldric. For the next 20 days development was similar, but there was a decrease in the rate of maturation after this, such that

Table 5.1
Changes in Main Morphological Features of Developing Barley Grain (data from Baxter[13])

Age Days	Size of Amyloplasts (um)	Morphological Description	Colour
2		Free nuclear stage	
3	1	Proplastids and Pro-mitochondria	
5	1	Formation of cell walls	
7	2	Turgid pearl like Endosperm Amyloplasts stain with iodine	
10-12	3-4	Watery endosperm	
14	3-4	Thin Sliver of Endosperm 3 - 4 mm	
15	5	Endosperm thickening	
18	6	Milky stage of endosperm embryo can be separated	
21	8	Aleurone layer can be scraped off endosperm	
25	10		
30	12	Chlorophyll layer begins to be reabsorbed	
33-35	15-17		
40	16	Endosperm begins to dry out Testa-pericarp adhering to endosperm	
45-50	15		
50-60	15-16	Grain shrinks due to water loss	

Colour: Bright green due to Chlorophyll layer of testa pericarp. → pale green → Turns Yellow beginning with awns.

Figure 5.1 Development of Grain



grain was ready for harvest 65 days after anthesis compared to 60 days for Maris Baldric.

The 1975 and 1976 crops showed a higher rate of development than Maris Baldric during the first 28 days after anthesis, after this the rate of development was similar. Thus, grain was ready for harvest on 51st day in 1975 and on the 49th day in 1976.

II PEPC, RBPC and Chlorophyll in Pericarps of Spring Grown Barley.

The total activity of PEPC, RBPC and chlorophyll content of the pericarps rose from an initial low value to a maximum and then fell again as the grain approached maturity (Fig. 5.2). The maximum value occurred at 25 days after anthesis for PEPC activity and chlorophyll content, and at 30 days after anthesis for RBPC activity, although at this stage of development the RBPC activity was not significantly greater than that recorded at 25 days after anthesis.

The time course of enzyme activity depends on whether activity is expressed on a chlorophyll basis or in total activity per grain. PEPC activity is approximately constant throughout grain development, although a small peak in activity occurred at 30 days after anthesis. In contrast to this, the activity of RBPC per mg chlorophyll was initially high and fell throughout development.

III PEPC and Chlorophyll in the Pericarps of Summer Grown Barley.

The relationship between PEPC activity and chlorophyll content with time for pericarps from barley in ear during July and August (1975) is shown in Fig. 5.3. The pattern of activity is similar to that for barley in ear in Spring but the maximum values (at twenty five days after anthesis) are very different. The maximum activity of PEPC was 197 nmoles CO_2/pericarp/min compared to 10.2 nmoles CO_2/pericarp/min in Spring. The maximum chlorophyll content of the pericarps of the barley in ear in Summer (3.9 µg/pericarp) was almost half that of

Figure 5.2 Changes in Enzyme Activity and Chlorophyll Content of Pericarps from Spring Grown Barley

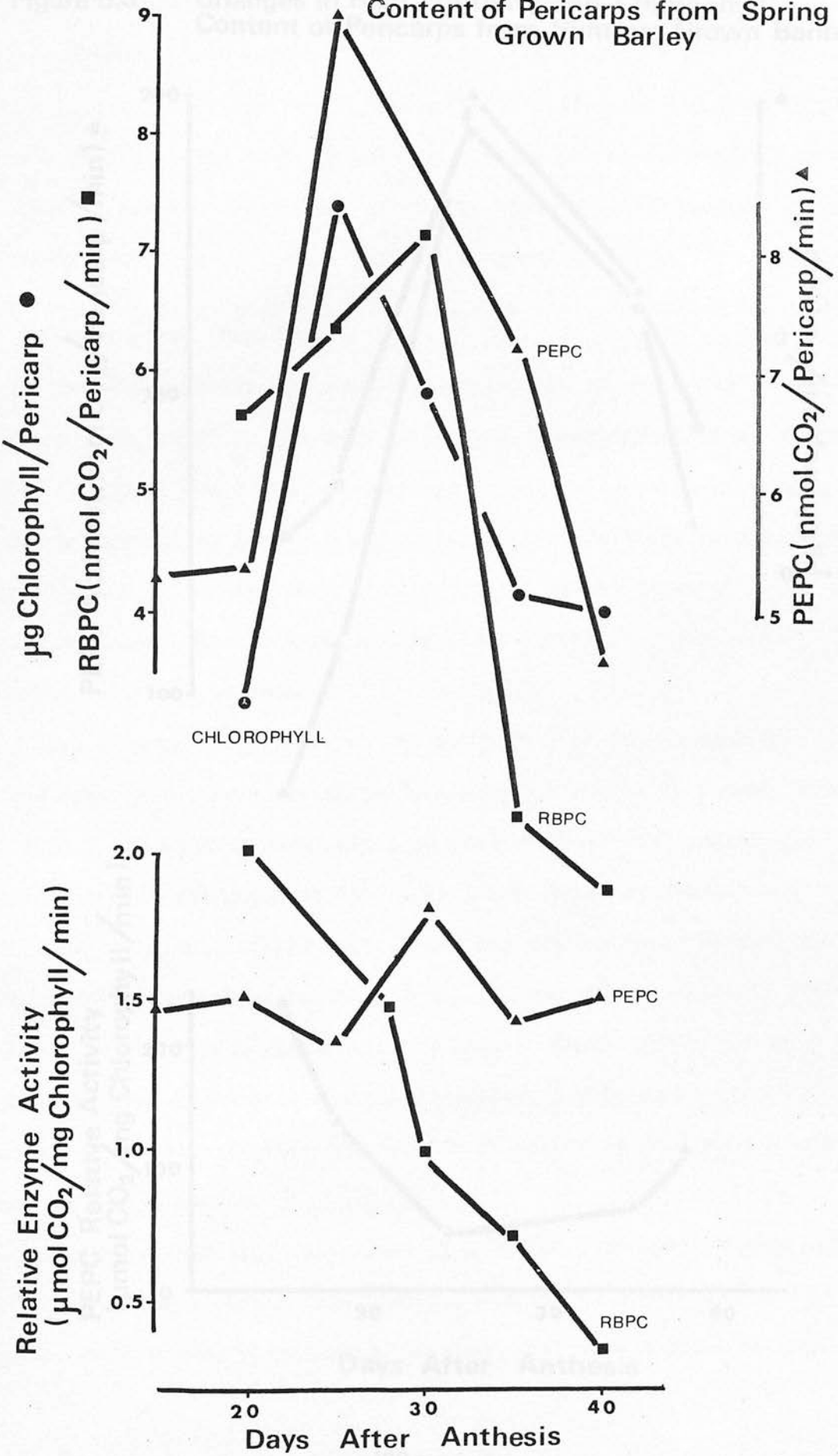
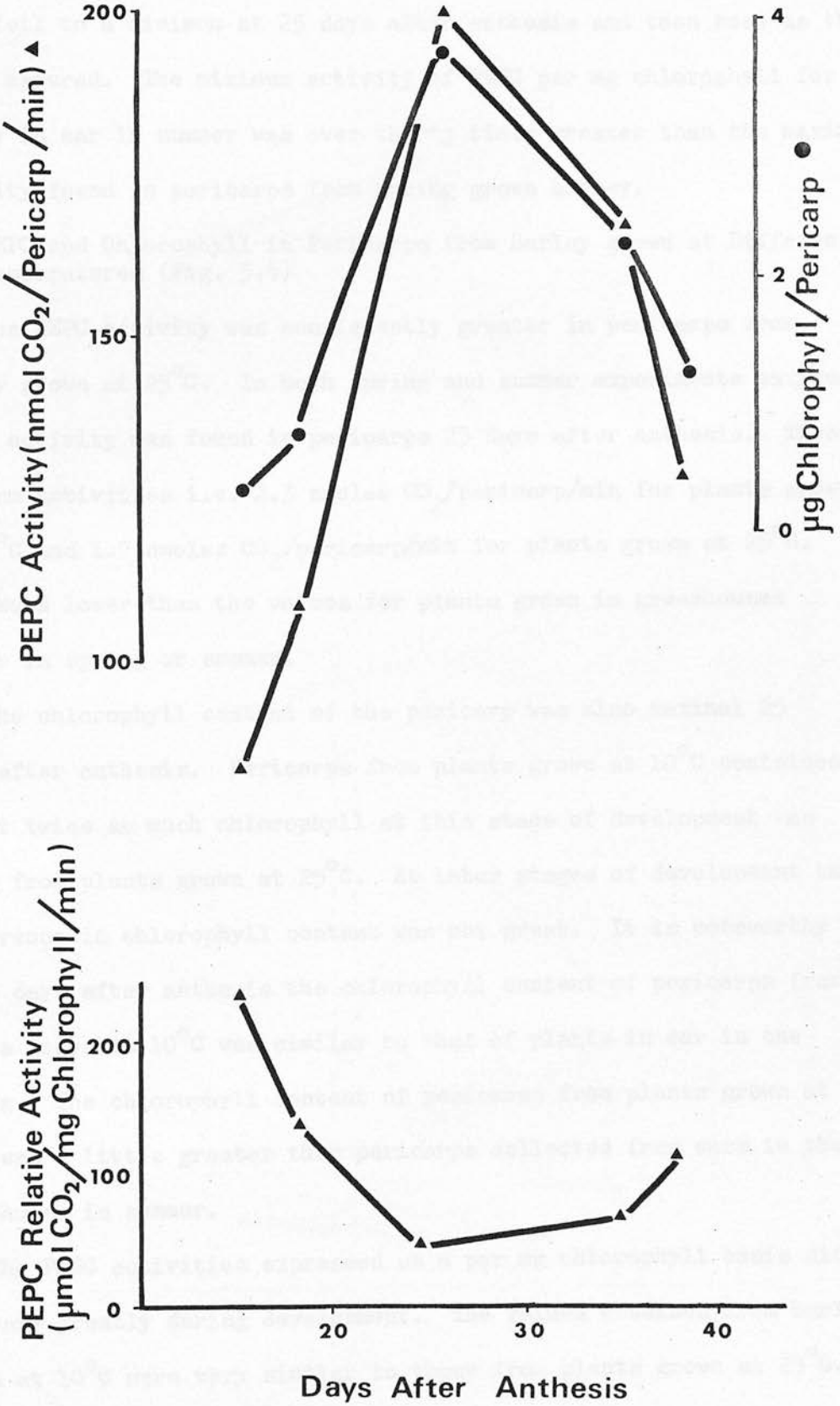


Figure 5.3 Changes in PEPC Activity and Chlorophyll Content of Pericarps from Summer Grown Barley



the barley in ear in Spring.

The measured activity of PEPC on a chlorophyll basis differs from those obtained from spring grown plants. Initially, activity was high this fell to a minimum at 25 days after anthesis and then rose as the grain matured. The minimum activity of PEPC per mg chlorophyll for barley in ear in summer was over thirty times greater than the maximum activity found in pericarps from spring grown barley.

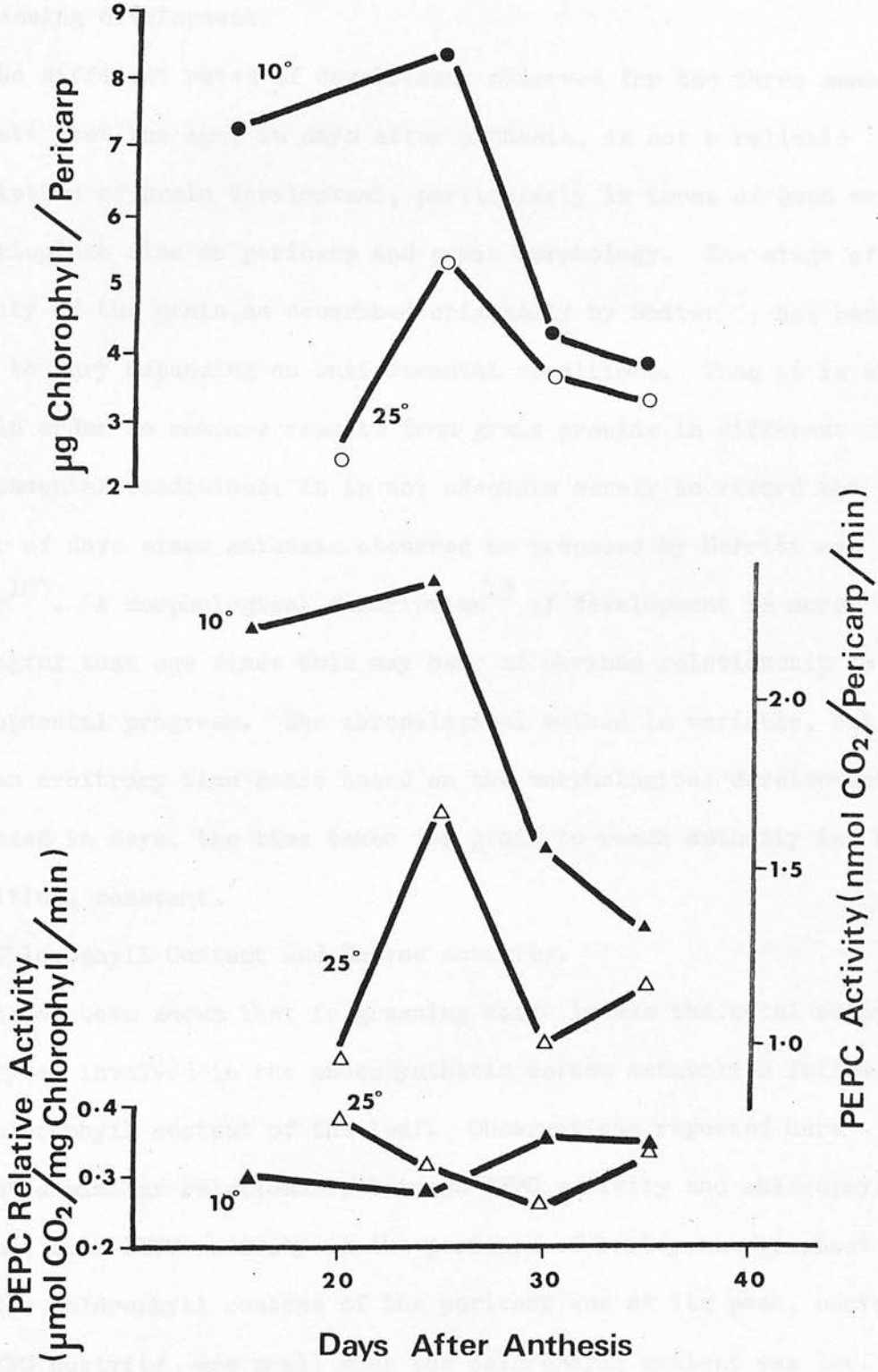
IV PEPC and Chlorophyll in Pericarps from Barley grown at Different Temperatures (Fig. 5.4)

The PEPC activity was consistently greater in pericarps from barley grown at 25°C. In both spring and summer experiments maximum PEPC activity was found in pericarps 25 days after anthesis. These maximum activities i.e. 2.3 nmoles CO_2/pericarp/min for plants grown at 10°C and 1.7 nmoles CO_2/pericarp/min for plants grown at 25°C, were much lower than the values for plants grown in greenhouses either in spring or summer.

The chlorophyll content of the pericarp was also maximal 25 days after anthesis. Pericarps from plants grown at 10°C contained almost twice as much chlorophyll at this stage of development as those from plants grown at 25°C. At later stages of development the difference in chlorophyll content was not great. It is noteworthy that at 25 days after anthesis the chlorophyll content of pericarps from plants grown at 10°C was similar to that of plants in ear in the spring. The chlorophyll content of pericarps from plants grown at 25°C was a little greater than pericarps collected from ears in the greenhouse in summer.

The PEPC activities expressed on a per mg chlorophyll basis did not vary greatly during development. The values obtained from barley grown at 10°C were very similar to those from plants grown at 25°C.

Figure 5.4 Changes in PEPC Activity and Chlorophyll Content of Pericarps from Barley Grown at 10°or 25°



The activity of PEPC per mg chlorophyll for pericarps from barley grown in the incubator (approximately 0.35 μmoles CO_2/mg chlorophyll/min) was less than a quarter of the activity found in greenhouse grown plants in the spring.

Discussion

I Assessing development.

The different rates of development observed for the three seasons indicate that the age, in days after anthesis, is not a reliable description of grain development, particularly in terms of such markers as amyloplast size or pericarp and grain morphology. The stage of maturity of the grain, as described originally by Baxter[13], has been found to vary depending on environmental conditions. Thus it is clear that in order to compare results from grain growing in different environmental conditions, it is not adequate merely to record the number of days since anthesis occurred as proposed by Merritt and Walker[103]. A morphological description[13] of development is more meaningful than age since this may bear no obvious relationship to developmental progress. The chronological method is variable, but with an arbitrary time scale based on the morphological development expressed in days, the time taken for grain to reach maturity is, by definition, constant.

II Chlorophyll Content and Enzyme Activity.

It has been shown that in greening maize leaves the total activity of enzymes involved in the photosynthetic carbon metabolism follows the chlorophyll content of the leaf. Observations reported here suggest a similar relationship between PEPC activity and chlorophyll content, i.e. PEPC activity in the pericarp of barley was greatest when the chlorophyll content of the pericarp was at its peak, conversely, the PEPC activity was small when the chlorophyll content was low.

Pericarp PEPC, therefore, may have a function in photosynthetic carbon dioxide fixation.

The activity of RBPC also followed the chlorophyll content of the pericarp. This is different from that described in the barley leaf where RBPC activity has been shown (in continuous light) to remain at a constant level, both on a per g fresh weight and a per mg protein basis[112].

III Effect of Environment on PEPC Activity

The simultaneous rise and fall of PEPC activity and chlorphyll content of the pericarp observed in all conditions of growth may indicate that the function of PEPC does not change with environment. On the other hand it has been shown that etiolated maize leaves fail to synthetise chlorophyll below 16°C when transferred to the light[101]. This together with the observation of Slack et al.[123] that low night temperatures can greatly reduce the activity of PEPC in leaves of C_4 plants, indicates that at low temperatues the metabolism of C_4 leaves becomes similar to that of C_3 leaves. Thus,it might be expected that pericarps grown in cold conditions would show less PEPC activity. Indeed the activity of PEPC was much greater in summer grown pericarps than in spring grown specimens, but the situation was reversed in incubator grown samples.

A better indication of the effect of environment upon PEPC activity may perhaps be gained from graphs of PEPC activity expressed on a per mg chlorophyll basis. These graphs show an almost constant relative PEPC activity throughout development apart from plants grown in greenhouses in summer, which have a minimum relative PEPC activity at the time when chlorophyll content of the pericarp is maximal. Also results obtained from summer grown plants and plants grown at 25°C in an incubator differed. These results may be explained if the PEPC

activity is light dependent. The light intensity in the incubator was much below that in the greenhouse in summer.

Conclusions

The method of Baxter[13] for determining the stage of development of grain provides a useful basis for comparison of grain produced in different environments where the time taken for grain to reach maturity may differ.

There is some evidence that activity of PEPC in the pericarp of barley may depend on environmental factors. To reach firm conclusions obviously further study is required using plants grown in a range of temperatures at various light intensities.

If the pericarps behave as the leaves of C_4 plants it is probable that there will be a minimum light intensity and temperature required for the development of high activities of PEPC.

Chapter 6

Carbon Dioxide Fixation

Introduction

The results described in previous chapters suggest that the pericarps of both wheat and barley have some unusual properties compared to the leaves. Enzyme studies (Chapter 3) certainly indicate that the pathway of carbon dioxide fixation may differ from that reported for the leaves[29].

One of the major difficulties in working with pericarps is the small quantity of tissue available. For example, Hatch and Slack[69] used six pieces of sugar cane leaf each 180 mm x 15 mm to identify products of carbon dioxide fixation. In order to obtain a similar amount of peri carp tissue, hundreds of grains would have to be dissected. Furthermore, previous work (Chapter 2) demonstrated the necessity for speedy preparation of isolated pericarps because of the presence of products of the phenol oxidase reaction. Another difficulty is that the pericarp tissue wilts rapidly on removal from the grain. Novel techniques have thus had to be devised in order to overcome these difficulties.

Preliminary experiments were carried out supplying homogenates and suspensions of pericarp with bicarbonate. In these experiments incorporation of C^{14} was not at a sufficiently high rate to allow the identification of products. In addition the technique of two dimensional paper chromatography used by Bassham et al.[12] to identify products of photosynthesis, was found to require much more radioactive material than was available following photosynthesis by isolated pericarps. The method described below used isolated pericarps on moistened filter paper discs. These were allowed to photosynthesise

in an atmosphere containing $C^{14}O_2$ and the products were separated using high voltage electrophoresis.

This chapter describes the metabolic events following carbon dioxide fixation by isolated pericarps. Results obtained from similar experiments with a range of plant materials are included for comparison.

Methods

I Plant Material

Plants were grown as described in Chapter 2. The green layer of the pericarp was peeled from grains of barley (var. Julia) 25 - 30 days after anthesis, and wheat (var. Maris Dove) at the stage of development most closely corresponding to that of barley 25 - 30 days after anthesis.

II Incubation of Isolated Pericarps, Grain and Leaf Discs.

The pericarps were placed on glass fibre discs, (21 mm diameter, 10 pericarps per disc) previously soaked in isotonic medium (50 mM - tricine KOH pH 7.5 and 0.33 M-sorbitol) to prevent dehydration of the tissues. In some experiments grains were used with the husk (palea and lemma) and the transparent layer of the pericarp removed, thus leaving the pericarp attached but intact. In such experiments three grains were placed on each moistened disc. Leaves of wheat, barley and pea (Pisum sativum var. Meteor), maize (Zea mays var. Golden Bantam) and Sedum spectabile were also studied. In these experiments, a disc of leaf material (5 mm diameter) was placed on each moistened glass fibre disc.

Three discs (holding isolated pericarps, intact pericarps on the grain or a piece of leaf) were placed in a perspex chamber (volume 11 ml) and 20 µl of a 17 mM solution of sodium (C^{14}) bicarbonate (specific activity approximately 60 Ci/Mole) was injected though a rubber seal into a well containing 0.15 ml of lactic acid, such that

the final concentration of CO_2 in the chamber was 0.1% by volume.

The chamber was illuminated at 25°C for periods of time ranging from 0.5 - 10 minutes with a 1 kW tungsten halogen lamp (incident light intensity 17,000 lux). In order to study the effects of darkness on products and rate of CO_2 fixation the chamber was placed in a covered bath at 25°C.

III Separation of the Products into Alcohol, Chloroform and Water Soluble Components.

The discs were transferred as quickly as possible to a Buchner funnel connected to a suction pump, and the tissue washed with water to remove sorbitol which interfered with the subsequent separation of radioactive products. The plant tissue was then transferred to a Griffiths' all glass hand held tissue grinder containing 3 ml of ethanol at 70°C and the tissue homogenised. The ethanolic suspension was then allowed to settle and the soluble fraction decanted into a 10 ml centrifuge tube. The solids were washed with two further 3 ml aliquots of hot ethanol. Both solids and ethanol were transferred to the centrifuge tube, and the mixture was centrifuged at 1,500 g for 2 min. The supernatant was transferred to a measuring cylinder and made up to 10 ml with ethanol. The concentration of chlorophyll was determined by the method of Wintermans and Demots[146] (see Chapter 3 for details).

The pellet was resuspended in 5 ml of water. The tube was covered and left for 24 hours at room temperature.

The alcoholic fraction was evaporated to dryness in a rotary evaporator. Lipids which may affect electrophoretic separation of the sugar phosphates formed in photosynthesis were removed by washing the residue with two 0.5 ml aliquots of chloroform. Three samples of 0.1 ml of chloroform extract were counted for radioactivity as

described in Chapter 2.

The solids remaining after washing with chloroform were again subjected to rotary evaporation to remove any remaining chloroform, and were then taken up in 0.25 ml methanol. Three 0.01 ml samples of the methanol extract were counted for radioactivity. The amount of radioactivity in water soluble compounds was determined after centrifugation (1,500 g for 2 minutes) of the aqueous suspension of alcohol insoluble solids. Three samples of volume 0.5 ml were counted. For these experiments the rate of CO_2 fixation was calculated from the sum of the total radioactivity in the chloroform, methanol and aqueous extracts. Rates were measured in nmol CO_2/min/mg chlorophyll.

In some experiments the pellet of water insoluble solids, consisting mainly of starch granules was washed twice further with water and then suspended in 5.0 ml of water and heated on a boiling water bath for 10 minutes to solubilise starch. The suspension was centrifuged for 5 minutes at 1,500 g and three 0.5 ml samples of the supernatant were counted for radioactivity.

IV Incorporation of ^{14} Carbon into the Endosperm.

Grain of barley Albino Lemma and var. Julia and wheat (var. Maris Dove) with the husk (palea and lemma), and transparent layer of the pericarp removed were incubated as described for 10 minutes. The green pericarp was then removed from the endosperm and aleurone layer and discarded. The endosperm including the aleurone layer were then homogenised in 3.0 ml of 1% sodium fluoride solution. Insoluble material was removed by centrifugation (10,000 g for 10 min) and then washed twice further with sodium fluoride solution. After each washing, samples (0.5 ml) of the supernatant were counted for radioactivity. The solid material was then made up to 3.0 ml with water and heated in a boiling water bath for 10 min to solubilise the starch. Material

which remained insoluble after heating was removed after centrifugation (10,000 g for 10 min). A sample (0.5 ml) of the boiling water extract was counted for radioactivity. The pellet was resuspended in water, heat treated, centrifuged and the supernatant estimated for radioactivity.

V Electrophoretic Separation.

Samples (0.05 ml) of the methanol extracts of plant tissues were subjected to electrophoretic separation as described by Farineau[51]. Three samples were spotted on to a piece of Whatman No. 1 chromatography paper (150 mm x 600 mm) 100 mm from the cathode. Standard radioactive compounds were also included in the electrophoretogram to aid identification of the separated products. The paper was moistened with pyridine: acetic acid buffer pH 4.5 (pyridine : acetic acid : water, 4 : 6 : 490) and a potential difference of 3.5 - 4.5 kV applied for 20 - 25 minutes. The electrophoretogram was then placed in an oven at 80°C and removed as soon as it was dry. It was then placed in contact with photographic film for 28 days. The developed film was used to locate compounds containing C^{14} on the electrophoretogram.

To provide additional verification of product identity, samples of the methanol extract were subjected to chromatographic separation in a medium containing phenol, water, acetic acid and M-ethylene diamine tetra acetic acid (740 : 260 : 10 : 1 respectively).

Areas of the electrophoretogram containing C^{14} were punched from the paper and placed in scintillation vials together with 5.0 ml scintillant (50 g napthaline and 6 g 2,5 diphenyloxazide per litre of 1,4 dioxan) and 0.5 ml water. Counting efficiency was 80%. The amount of radioactivity in each compound was expressed as a percentage of the total radioactivity recovered from the electrophoretogram.

VI pH Changes in Grain after Incubation for Twenty-Four Hours in Light or Darkness.

Barley plants (var. Julia) twenty four days after anthesis were exposed to a twenty four hour day length by extending the period of illumination of mercury vapour lamps. Similar plants were subjected to a twenty-four hour dark period by placing them in an incubator set at the temperature of the greenhouse ($15^{o}C$). After this period three samples of 15 grains from plants subjected to each treatment were collected and after removal of the husk, were quickly homogenised in water (5 grain/ml) by hand with a Griffiths' all glass tissue grinder. The pH of the resultant suspension was then measured.

Results

I Rates of Carbon Dioxide Fixation by Various Plant Tissues.

The rates of $C^{14}O_2$ fixation on a chlorophyll basis are shown in Table 6.1. The highest rates were recorded for Sedum and maize leaves. On a chlorophyll basis pea leaves fixed carbon dioxide at twice the rate of barley leaves. Wheat leaves had a much lower rate of carbon dioxide fixation. The isolated pericarps of wheat and barley showed rates approximately equal to the respective leaves. Grain with the husk (palea and lemma) and transparent layer of the pericarp removed (the pericarps were therefore intact, each enclosing their endosperm and embryo), showed much higher rates of carbon dioxide fixation than isolated pericarps. The rates of carbon dioxide fixation in the dark both of wheat (isolated) and barley (intact) pericarps were very low.

II Products of ^{14}-Carbon Dioxide Fixation in the Light.

The distributions of C^{14} between the alcoholic, aqueous and chloroform extracts of plant material following incubation in $C^{14}O_2$

Table 6.1 Rates of Carbon Dioxide Fixation by Various Tissues.

Plant Source	Tissue	Condition Light (L) Dark (D)	Rate nMoles $^{14}CO_2$/mg Chlorophyll/min
Barley (var. Julia)	Leaf	L	17 ± 9
"	Isolated Pericarp	L	16 ± 6
"	Pericarp on Endosperm	L	43 ± 8
"	"	D	0.21 ± 0.06
Wheat (var. Maris Dove)	Leaf	L	0.9 ± 0.1
"	Isolated Pericarp	L	0.6 ± 0.1
"	"	D	0.2 ± 0.08
"	Pericarp on Endosperm	L	59 ± 2
Maize	Leaf	L	127 ± 19
Sedum	Leaf	L	130 ± 80
Pea	Leaf	L	34 ± 10

Figures are averages of five determinations $\pm$ standard deviation.

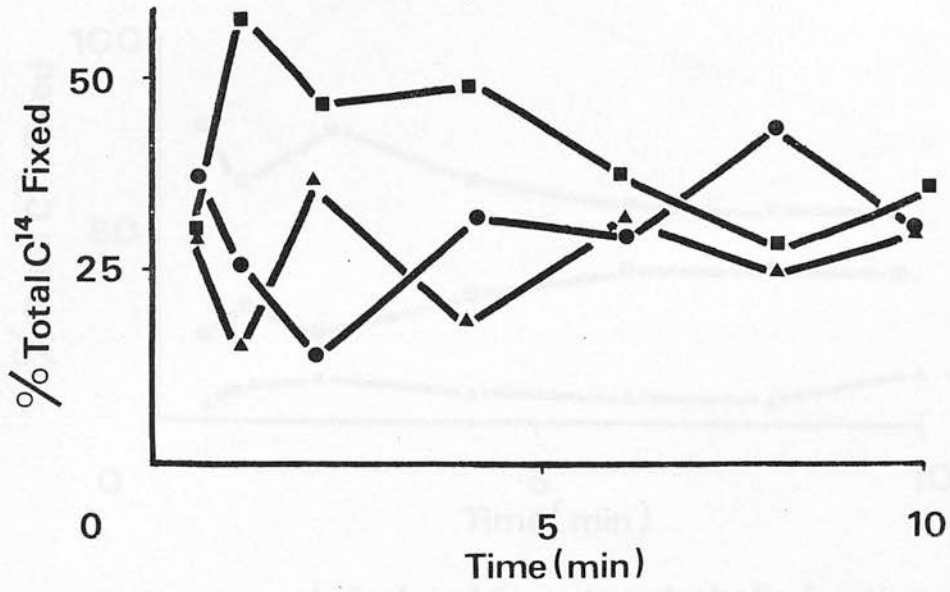
are shown in Figs. 6.1 - 6.11. The amount of C^{14} which was found in starch is also shown (Figs. 6.7 and 6.9). Where separation was effected the distribution of C^{14} between compounds isolated from the alcoholic fraction is also shown. Electrophoretic separation was not effective on alcoholic extracts of intact attached pericarps. This was probably due to the alcohol soluble constituents of the endosperm and embryo saturating the pyridine-acetic acid buffer.

The distribution of radioactivity between the chloroform and aqueous extracts was intended to give an estimate of the amount of C^{14} fixed in lipids and oligosaccharides. The alcoholic fraction was shown to contain the low molecular weight products of carbon dioxide fixation. There was often marked variation in the distribution of radioactivity between the fractions. This was the case for aqueous and alcoholic extracts of isolated wheat pericarps (Fig. 6.8). Thus, despite the care taken, the extraction of alcohol soluble compounds was not always complete. However, in most of the experiments, the distribution of C^{14} followed a distinct trend.

With the exception of the barley leaf (Fig. 6.4) and isolated barley pericarps (Fig. 6.6) which fixed C^{14} mostly into ethanol soluble compounds the aqueous fraction contained the highest fraction of radioactivity. All tissues fixed only a small amount of C^{14} into compounds taken up in chloroform except in the maize leaf (Fig. 6.1) where approximately equal amounts of C^{14} were extracted into the alcohol and chloroform fractions. The amount of C^{14} recovered in starch from intact wheat pericarps (Fig. 6.9) was approximately a constant fraction of the total fixed. For intact barley pericarps (Fig. 6.7), the amount of C^{14} recovered in starch rose with time, being less than 10% at 0.5 min but 34% after 10 minutes.

For most tissues the amount of C^{14} found in PGA, hexose monophos-

Figure 6.1 Distribution of C^{14} Fixed by Maize Leaf
a) between alcoholic ●, aqueous ■, and chloroform ▲ fractions



b) between compounds isolated from the alcoholic fraction

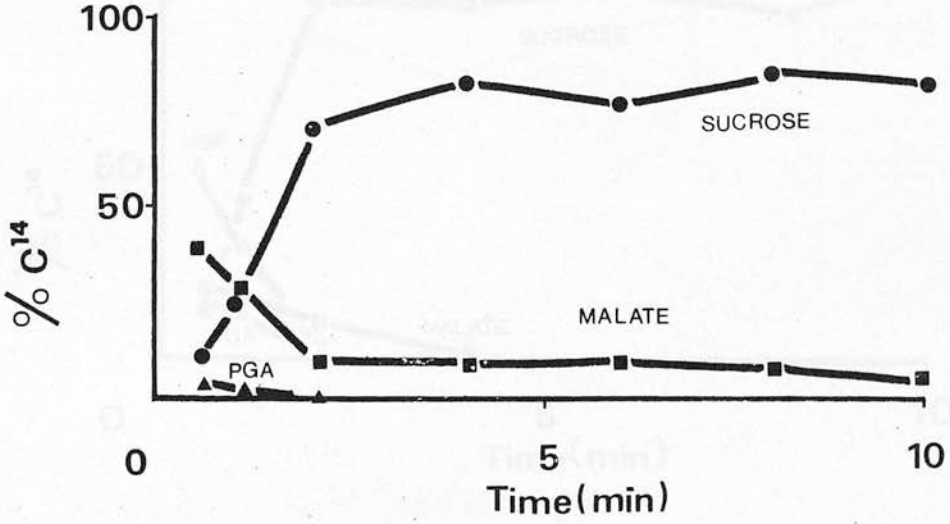
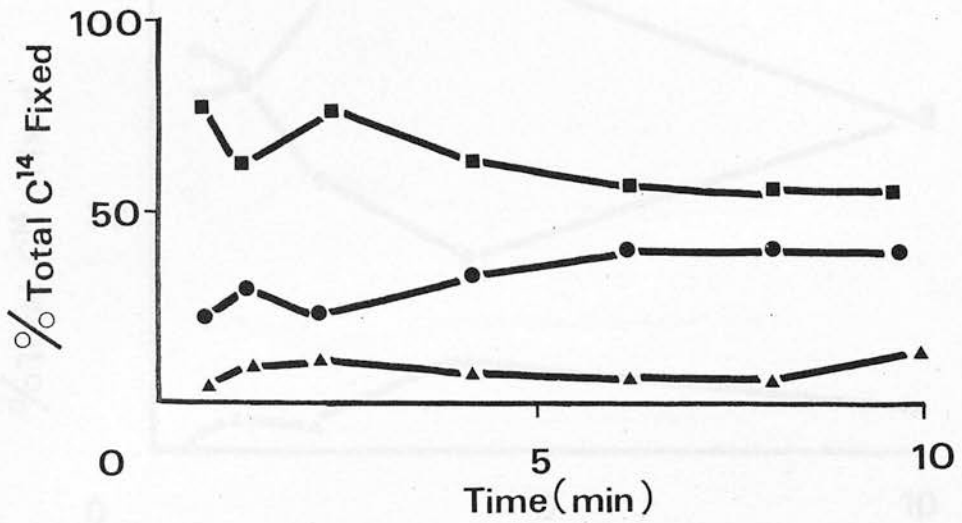


Figure 6.2 Distribution of C^{14} Fixed by Pea Leaf
a) between alcoholic ●, aqueous ■, and chloroform ▲ fractions



b) between compounds isolated from the alcoholic fraction

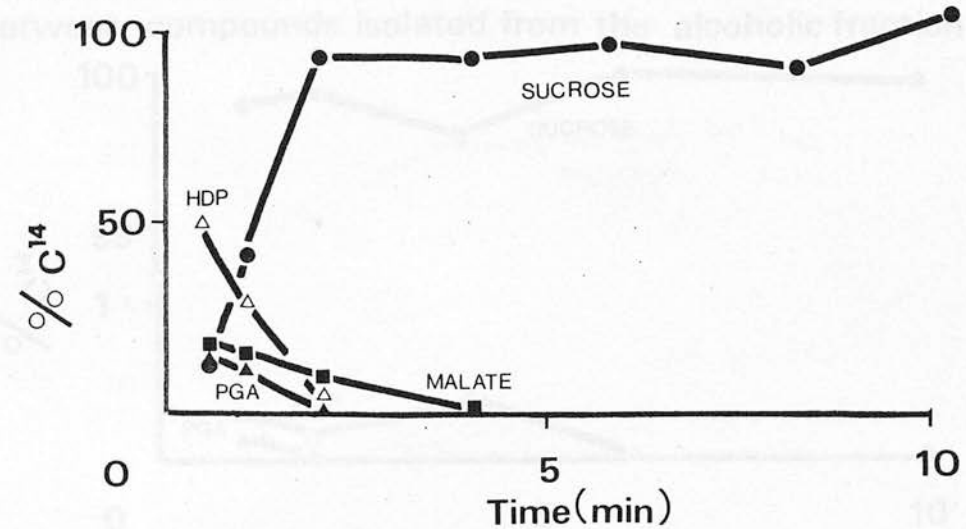
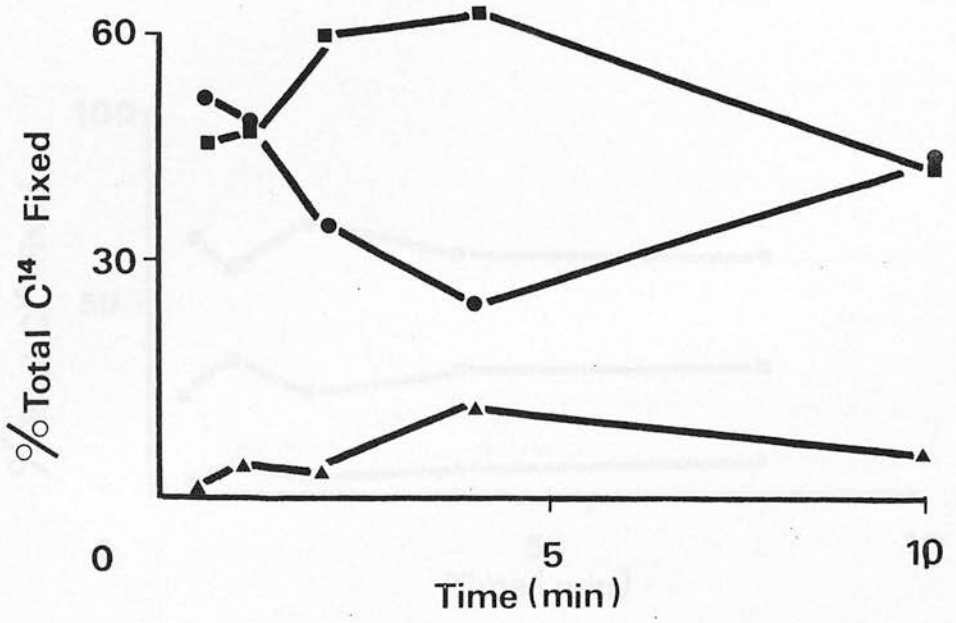


Figure 6.3 Distribution of C^{14} Fixed by Sedum Leaf
a) between alcoholic ●, aqueous ■, and chloroform ▲ fractions



b) between compounds isolated from the alcoholic fraction

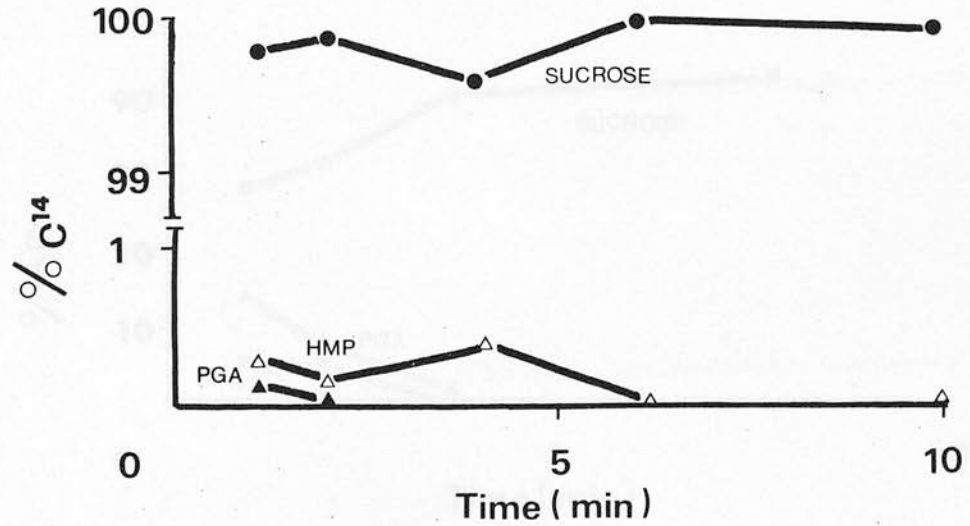
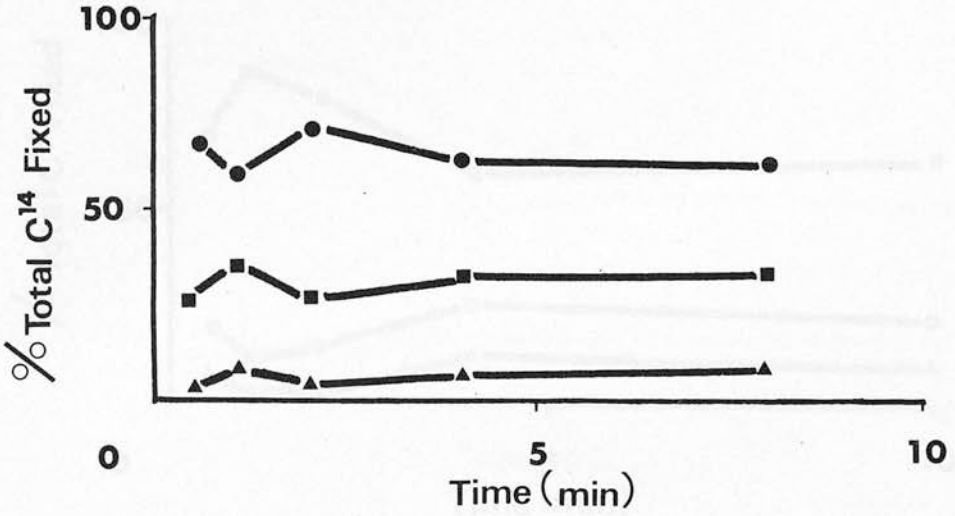


Figure 6.4 Distribution of C^{14} Fixed by Barley Leaf
a) between alcoholic ●, aqueous ■, and chloroform ▲ fractions



b) between compounds isolated from the alcoholic fraction

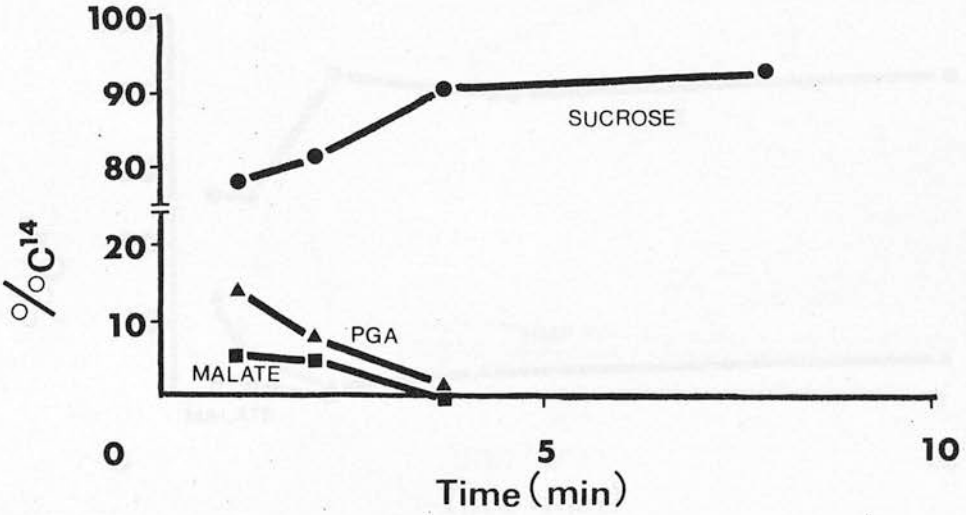
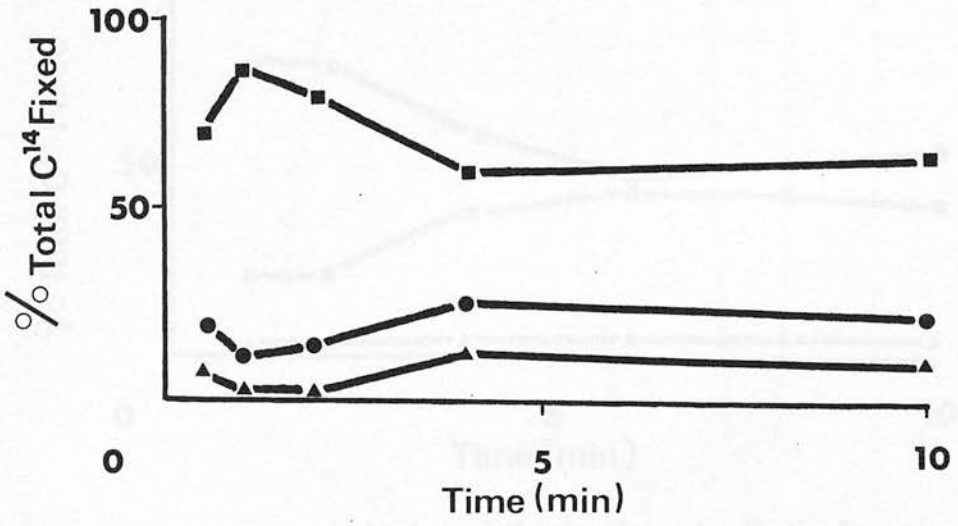


Figure 6.5 Distribution of C^{14} Fixed by Wheat Leaf
a) between alcoholic ●, aqueous ■, and chloroform ▲ fractions



b) between compounds isolated from the alcoholic fraction

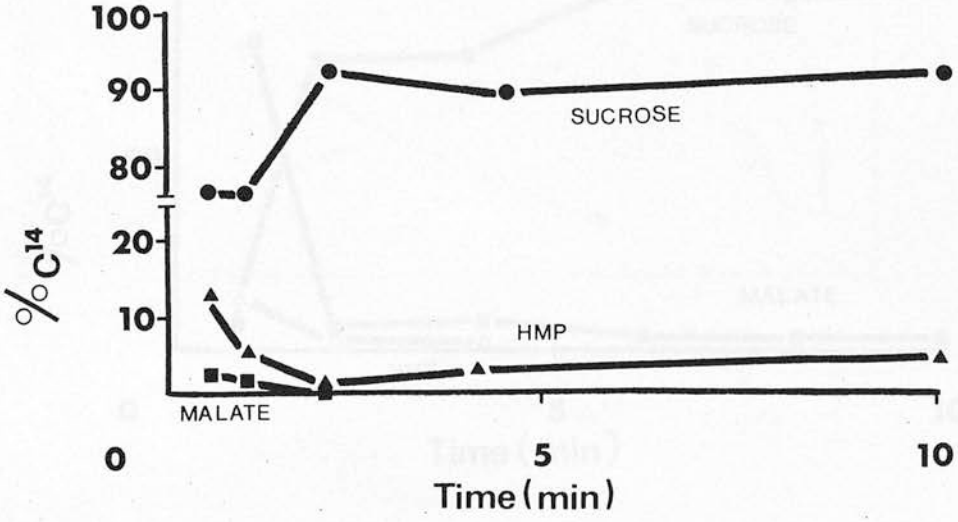
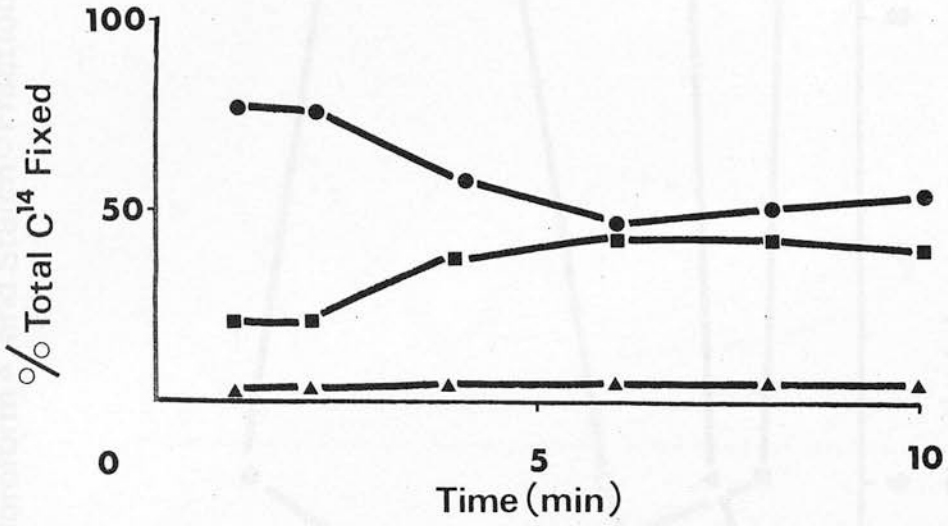


Figure 6.6 Distribution of C^{14} Fixed by Isolated Barley Pericarps
a) between alcoholic ●, aqueous ■, and chloroform ▲ fractions



b) between compounds isolated from the alcoholic fraction

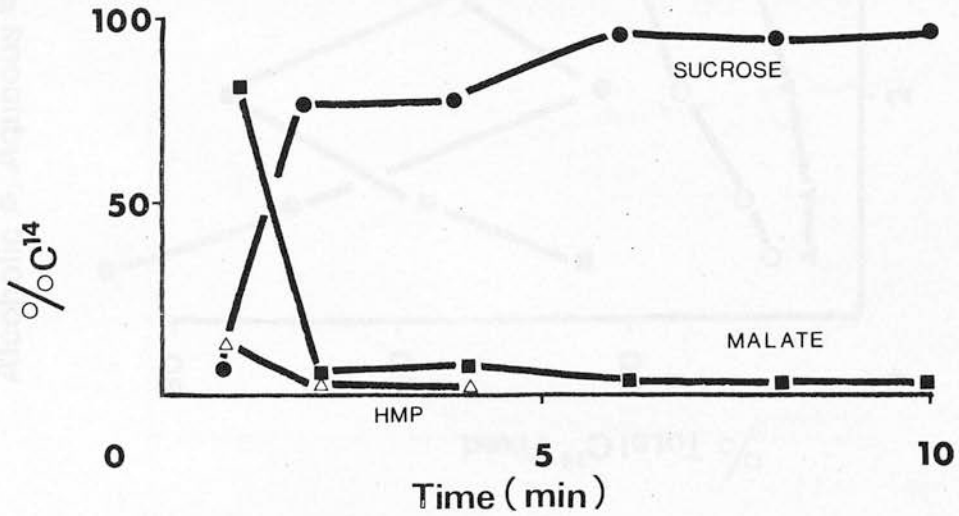


Figure 6.7 Distribution of C^{14} Fixed by Intact Barley Pericarps between Alcoholic ●, Aqueous ■, Chloroform ▲, and Starch ○ Fractions

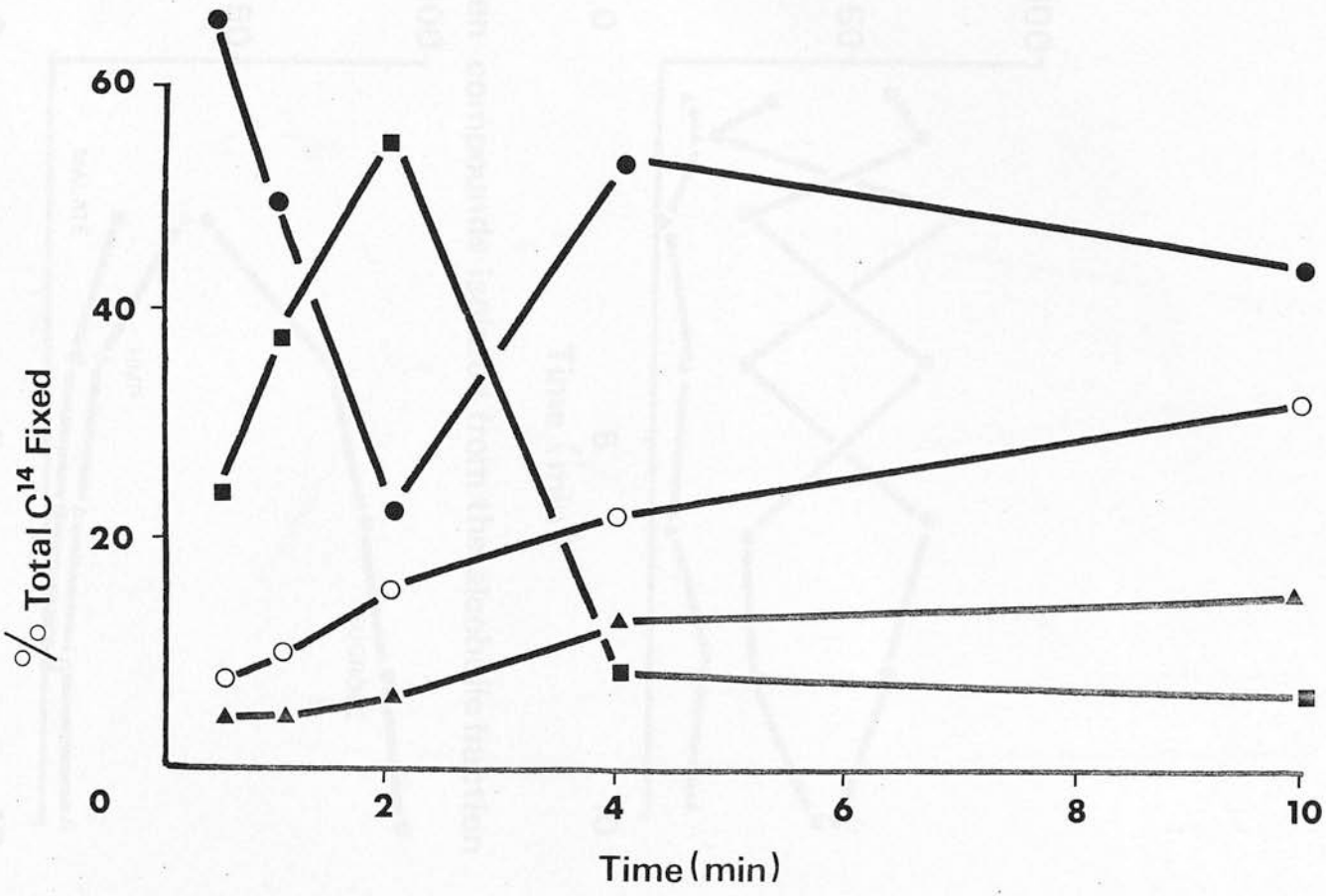
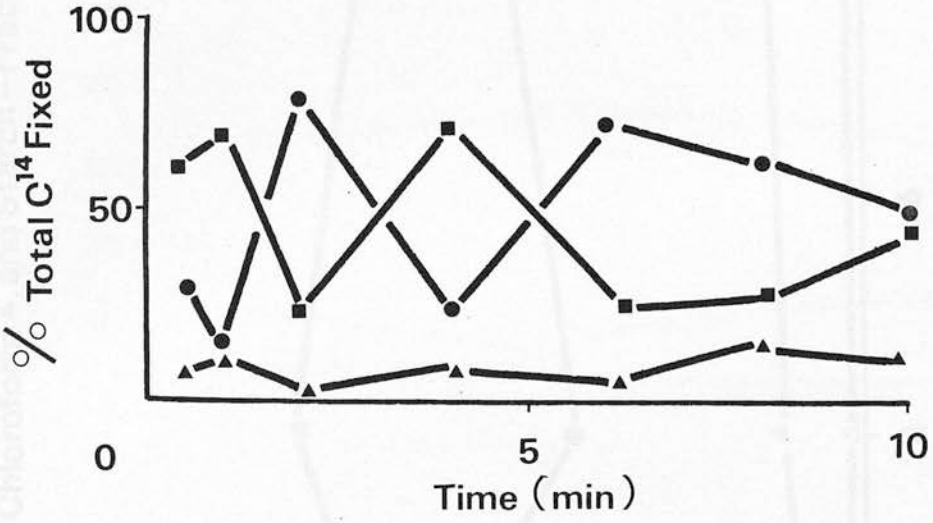


Figure 6.8 Distribution of C^{14} Fixed by Isolated Wheat Pericarps
a) between alcoholic ●, aqueous ■, and chloroform ▲ fractions



b) between compounds isolated from the alcoholic fraction

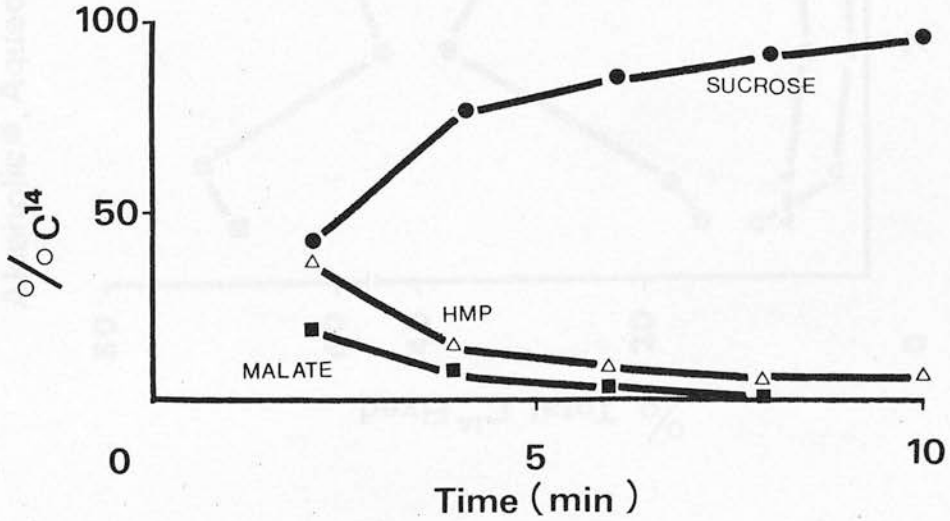


Figure 6.9 Distribution of C^{14} Fixed by Intact Wheat Pericarps between Alcoholic ●, Aqueous ■, Chloroform ▲, and Starch △ Fractions

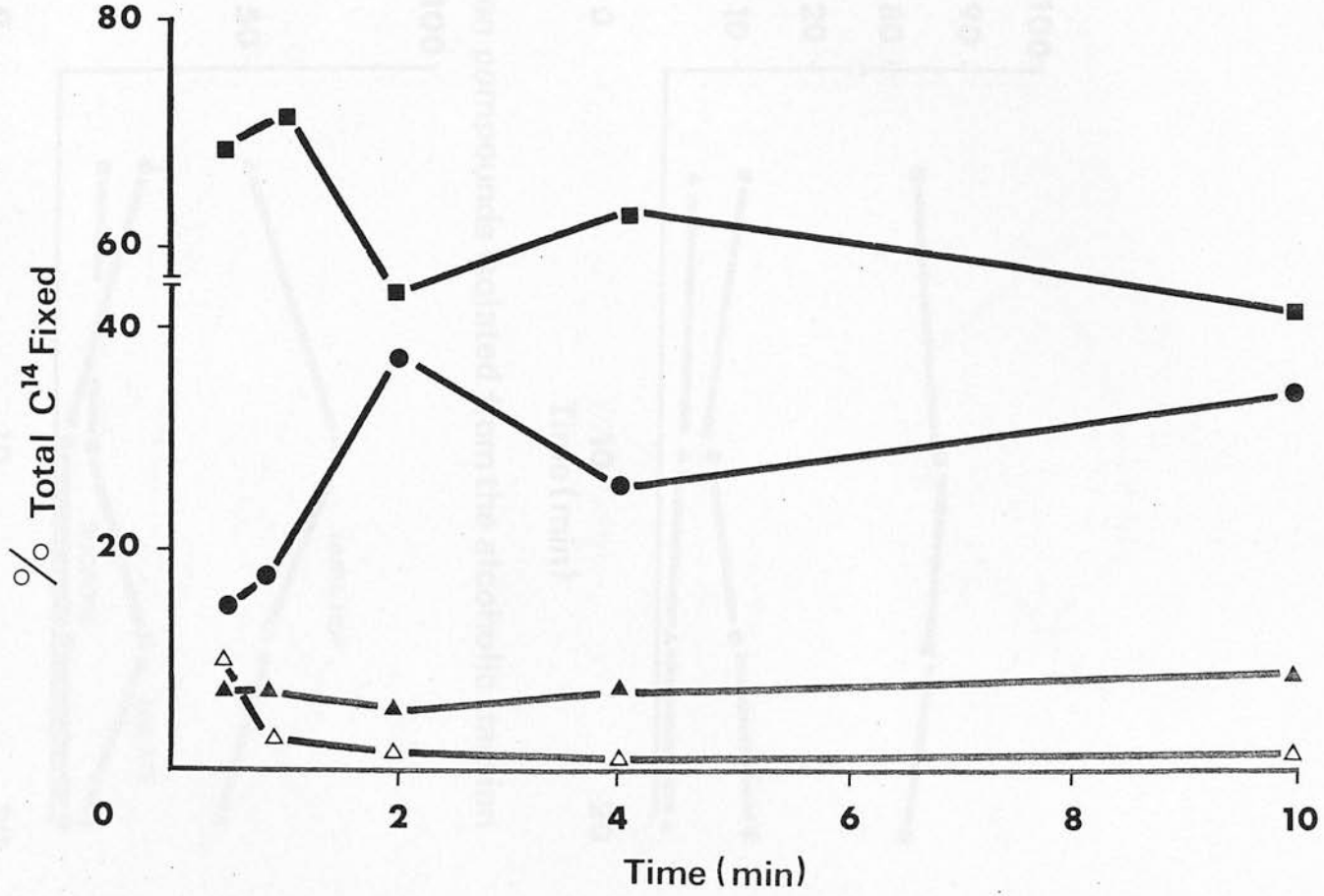
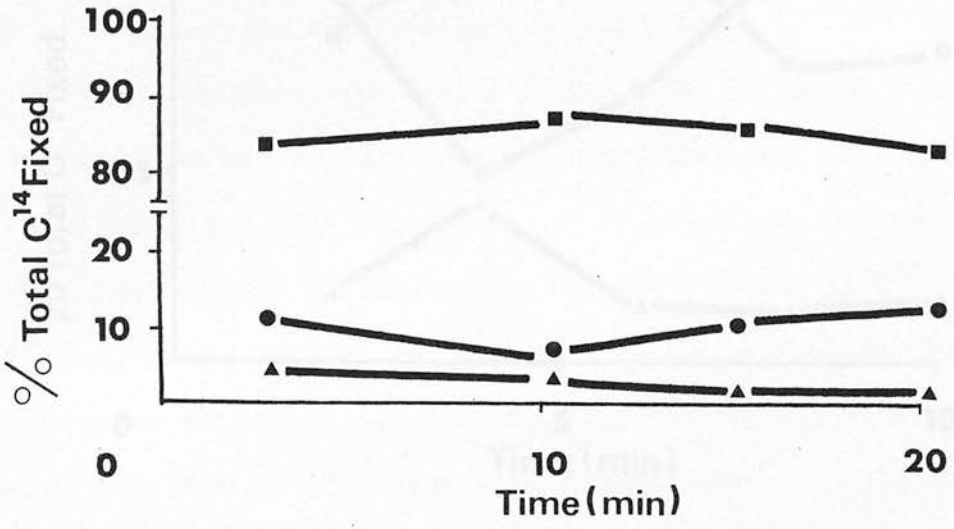


Figure 6.10 Distribution of C^{14} Fixed by Intact Barley Pericarps in the Dark a) between alcoholic ●, aqueous ■, and chloroform ▲ fractions



b) between compounds isolated from the alcoholic fraction

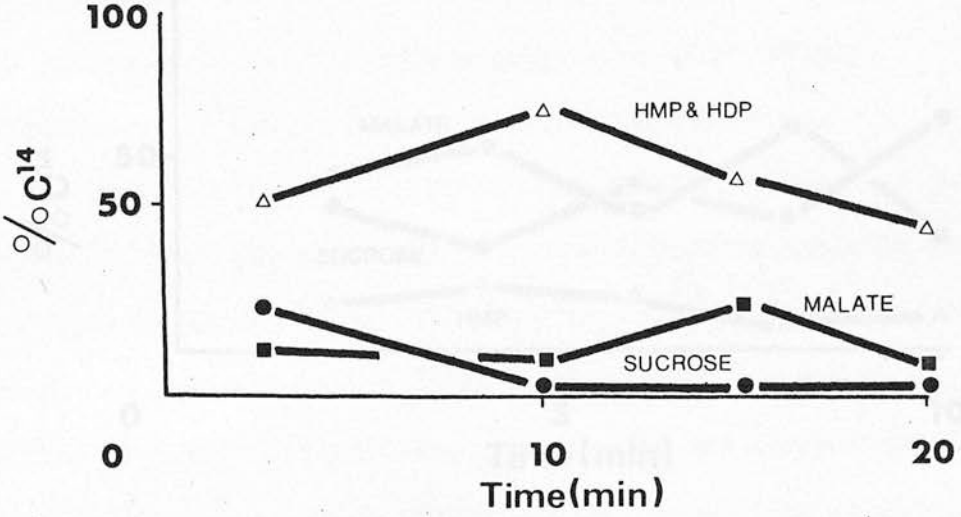
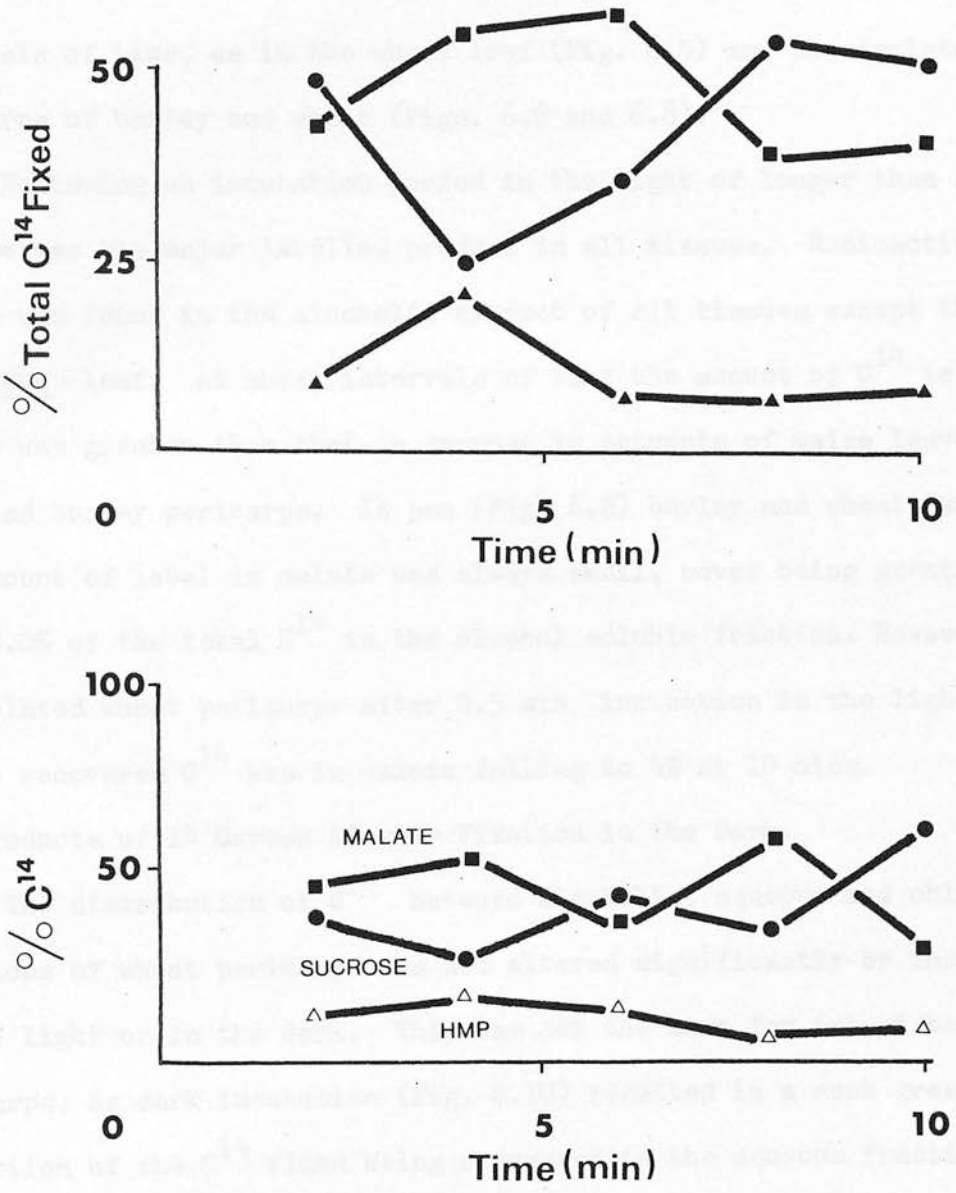


Figure 6.11 Distribution of C^{14} Fixed by Isolated Wheat Pericarps in the Dark a) between alcoholic ●, aqueous ■, and chloroform ▲ fractions



phate(HMP) and hexose diphosphate (HDP) was small compared with the total recovered after electrophoresis of the alcohol soluble fraction. PGA, HMP, and HDP are not shown in the figures 6.1 - 6.8 unless the amount of label present in these compounds was significantly greater than zero. Significant amounts of radioactive PGA were found after short incubation periods in Sedum and barley leaves (Figs. 6.3 and 6.4). The fraction of C^{14} recovered in HMP was generally higher at short intervals of time, as in the wheat leaf (Fig. 6.5) and the isolated pericarps of barley and wheat (Figs. 6.6 and 6.8).

Following an incubation period in the light of longer than 2 min sucrose was the major labelled product in all tissues. Radioactive malate was found in the alcoholic extract of all tissues except that of the Sedum leaf. At short intervals of time the amount of C^{14} in malate was greater than that in sucrose in extracts of maize leaves and isolated barley pericarps. In pea (Fig. 6.2) barley and wheat leaves the amount of label in malate was always small, never being greater than 8.0% of the total C^{14} in the alcohol soluble fraction. However, in isolated wheat pericarps after 0.5 min incubation in the light 24% of the recovered C^{14} was in malate falling to 4% at 10 mins.

IV Products of ^{14} Carbon Dioxide Fixation in the Dark.

The distribution of C^{14} between alcoholic, aqueous and chloroform fractions of wheat pericarps was not altered significantly by incubation in the light or in the dark. This was not the case for intact barley pericarps, as dark incubation (Fig. 6.10) resulted in a much greater proportion of the C^{14} fixed being recovered in the aqueous fraction. For both wheat and barley pericarps the dark incubation resulted in a much reduced production of labelled sucrose. The proportion of label recovered in hexose phosphates (HMP and HDP) was much greater in the dark than in the light. For wheat pericarps incubated in the

light for short periods of time, a high proportion of the C^{14} fixed (approximately 40%) was found in hexose phosphate as the incubation period increased the amount of label in hexose phosphate fell (e.g. to 3% at 10 min). The fraction of label in hexose phosphate was nearly constant for dark incubated wheat pericarps (8 - 18%).

V Transfer of 14-Carbon from Pericarp to Endosperm.

The amount of C^{14} recovered in endosperm starch from barley and wheat after 10 min incubation, is compared with that of similarly treated Albino Lemma in Table 6.2. Barley endosperm contained five times the amount of C^{14} found in the Albino Lemma endosperm.

VI The pH of Grain after Light and Dark Treatments.

The pH of homogenates from grain kept either in light or dark for twenty-four hours was not significantly different, 6.62 ± 0.04 after dark treatment, 6.57 ± 0.02 after light treatment.

Discussion

I Rates of 14-Carbon Dioxide Fixation.

It is evident from the differences in rates of $C^{14}O_2$ fixation in light and dark that CO_2 fixation by pericarps of wheat and barley is a light dependent process. The rates of photosynthetic CO_2 fixation of isolated and attached pericarps are very different. Photosynthetic oxygen evolution was also much greater in attached pericarps (Chapter 4). The low rate of oxygen production of isolated pericarps was attributed to tissue damage incurred during isolation procedures. This could also explain the lower rates of CO_2 fixation by isolated pericarps. It was shown in Chapter 4 that the endosperm and embryo have high rates of respiration. It is probable that in the experiments on grain, with husk (palea and lemma) and transparent layer of the pericarp removed, a large amount of the CO_2 fixed by the green layer of the pericarp originates from respiration by the endosperm and embryo. The rate of

Table 6.2 Transfer of C^{14} from Pericarp to Endosperm

Grain	Amount of C^{14} Recovered in the Endosperm (pM per grain)
Barley Var. Albino Lemma	0.5 ± 0.4
Barley Var. Julia	2.6 ± 0.3
Wheat Var. Maris Dove	10.1 ± 2.3

Figures are averages of three estimates $\pm$ standard deviation.

CO_2 fixation was estimated by measuring the amount of external $C^{14}O_2$ fixed. Any $C^{12}O_2$ fixation resulting from internal respiration was not included. Hence the overall rate of CO_2 fixation was no doubt underestimated.

In the experimental conditions described here the maize leaf had a rate of photosynthesis similar to that of Sedum but much greater than that of the other tissues. This is consistent with the findings of other workers, notably El-Sharkaway and Hesketh[47], who found higher fixation rates in C_4 compared to C_3 plants. The observation that the possibly underestimated rate of photosynthesis of attached pericarps was greater than that of the leaves of C_3 plants (barley, wheat and pea) is a further indication that the pericarp may have similar properties to C_4 or CAM plants.

II Separation of the Products of 14 - Carbon Dioxide Fixation.

The presence of label in the chloroform extracts gives a measure of the amount of lipids synthesised during CO_2 fixation. In most tissues this accounted for only a small amount of the total C^{14} fixed. However in the maize leaf approximately 30% of the total radioactivity was found in the chloroform fraction. As maize was the only classical C_4 plant studied it is not possible to ascertain if this is typical of C_4 plants. Other workers[84, 69] did not extract with chloroform and thus this has not previously been reported. Since larger molecules such as oligosaccharides tend to be insoluble in ethanol[8], it was hoped that extraction of plant material with ethanol followed by extraction with water would give some indication of the amount of C^{14} incorporated into short chain polysaccharides. Other workers have employed similar techniques in the analysis of leaf material. Hatch and Slack[69] combined alcoholic and aqueous washings prior to counting radioactivity, Kortshak et al.[84] used separate

alcoholic and aqueous washings but did not comment on the distribution of label between the two. In many of the tissues studied here, a high proportion of the C^{14} fixed was recovered in the aqueous fraction. This may indicate either a rapid formation of oligosaccharides in these tissues or that the extraction with alcohol was incomplete. It was assumed that the electrophoretic separation of compounds in the alcohol extract was representative of the compounds formed by photosynthetic CO_2 fixation, and that further extraction would not have altered the results.

The work reported here with pericarps differs in two respects from that described elsewhere using other tissues. Firstly, other workers have been able to separate products of photosynthesis after shorter intervals of time. Secondly, the concentration of CO_2 used in the present work was increased from 0.03% to 0.1% to ensure sufficient incorporation of C^{14} for the identification of early products of photosynthesis. It has been reported[32] that high concentrations of CO_2 result in saturation of C_4 metabolism such that the products of photosynthesis are similar to those of a C_3 plant. Hence the products of photosynthesis in tissues other than pericarps were determined in order to establish that the experimental conditions did not result in atypical products. In the present work, the time course of products found in the alcohol soluble fraction of maize leaves (Fig. 6.1) is very similar to that reported for C_4 plants by other workers[69,84]. The products of photosynthesis in the pea leaf are a little different from those reported by other workers[29]. After short periods of time malate is the second major product, however the amount of C^{14} in malate is small compared to that in sucrose. The labelled malate may result from catabolism of labelled sucrose or by a carboxylation reaction which is of little significance in low

concentrations of CO_2.

The distribution of ^{14}C in compounds isolated from the alcoholic extract of isolated pericarps was similar to that of maize leaves and to that of other C_4 plant leaves[69,84], in which malate was also the stable initial product of carbon dioxide fixation. Thus, while the barley pericarp may not have the anatomical properties thought to be characteristic of classical C_4 plants, it is capable of C_4 photosynthesis. Similar results were recorded in isolated wheat pericarps. However, due to the difficulty in obtaining sufficient incorporation of C^{14} to identify products at short intervals of time, the evidence is not as conclusive as in the case of barley.

As was discussed in Chapter 1, a survey of enzyme activity in the pericarp of developing barley[41] yielded results which were consistent with this tissue having C_4 photosynthesis of the NADME type[68]. However, the C_4 acid produced in greatest amounts by this type of C_4 plant is aspartic acid, while the results presented here show that malic acid is the C_4 acid produced in greatest amounts by the barley pericarp. This tissue must therefore be excluded from the NADME classification. This is a further indication of the singular nature of the cereal pericarp: that is the enzyme content is consistent with a plant of the NADME type, but the products of $C^{14}O_2$ fixation indicated that the tissue is of the NADPME type[68].

Although the evidence obtained from these experiments points to the presence of a C_4 pathway in isolated pericarps in the light, it is worth considering the possibility that malate formation is unrelated to, and distinct from the reactions of photosynthesis. If this was the case, the sugar phosphates resulted from the Calvin cycle activity then, while malate would become labelled, no transfer of label from malate to PGA, and therefore to sugar phosphates and sucrose, would

be observed. Such transfer is of course observed in C_4 photosynthesis[67,52]. If the formation of labelled malate was independent of the Calvin cycle then turning off the light should not affect the rate of incorporation of C^{14} into malate. It may be calculated from the results reported here, however, that for intact attached barley pericarps the rate of malate formation was over five hundred times that observed in the dark. It would thus appear that the formation of malate in barley pericarps is linked to photosynthesis. In isolated wheat pericarps on the other hand the amount of malate formed in the dark was marginally greater than in the light. This result does not necessarily exclude the possibility of an active C_4 pathway in the wheat pericarp. In the light the formation of malate and the subsequent transfer of CO_2 to PGA may be so efficiently linked that the rate of malate formation is equal to its decarboxylation. If this is the case in wheat, an explanation of the excess of malate formation over decarboxylation in barley must be found. It is possible that the barley pericarp incorporates some of the properties of the Crassulaceae and forms a pool of C_4 acid for subsequent transfer to the Calvin cycle when the supply of CO_2 becomes limited.

III Absence of Crassulacean Acid Metabolism in Barley Pericarps.

Although the rates of $C^{14}O_2$ fixation, in barley and wheat pericarps, into malate in the dark were small, it is possible that this may be the result of Crassulacean Acid type metabolism. The malate found in pericarps may have been fixed by a dark reaction in the short period prior to illumination. This is unlikely however as no such malate formation was observed in Sedum leaves. In the Crassulaceae a fall in the pH of leaf homogenates is observed following the onset of darkness[181]. No pH fall was observed in barley grain. It is always possible however that this might have been due to the buffering

capacity of this tissue.

IV The Transfer of Photosynthetic Products from the Pericarp to the Endosperm.

It is clear that the pericarp is capable of significant rates of photosynthesis. However, it has not yet been established whether the products of photosynthesis by the pericarp are available to the grain and stored there as carbohydrate in endosperm starch granules.

The amount of C^{14} found in the heat solubilised solids (derived from starch granules) of the endosperm of Albino Lemma barley was small compared to that of wheat and barley. As the grain of Albino Lemma without husk or transparent layer contains no tissue capable of photosynthesis, this incorporation may result from non-photosynthetic CO_2 fixation. The greatest amount of C^{14} was recovered from wheat endosperm which indicates an efficient transfer of photosynthetic products from the pericarp. The rate of photosynthesis by isolated wheat pericarps was nearly a hundred times less than that of the intact pericarp attached to the endosperm. Removal of the pericarp from the endosperm may damage the transport system, and cause a build up of products within the pericarp. Hence, photosynthesis may be inhibited.

Conclusions

The observed rates of CO_2 fixation together with the products suggest that photosynthesis in pericarps resembles that in C_4 plant leaves.

Chapter 7

Kinetics of PEPC Activity

Introduction

Leaves of C_3 plants have a low yet significant activity of the enzyme PEPC[92]. This enzyme has some properties similar to the PEPC found in etiolated leaves of C_4 plants[134]. For example both enzymes are stable to heat[92].

It is clear, however, that the properties of PEPC vary depending on the species and stage of development of the tissue. The PEPC from green sugar cane leaves (C_4) was shown to be activated by glucose 6-phosphate (G6P), while the enzyme from etiolated leaves was not[60]. A positive response to G6P has also been reported with PEPC from Crassulacean plants[135]. On the other hand it has been reported that the enzyme from etiolated maize can be activated by G6P[149]. Glycine also activates PEPC from green maize leaves but the effect is not observed in other C_4 plants[108].

Thus, the properties of PEPC vary depending on the species and stage of development of the tissue under examination.

Ting and Osmond[134] found the Michaelis Constant (Km) of PEPC with respect to PEP to be 0.14 mM in the leaves of C_3 and CAM plants, which is similar to that for etiolated C_4 leaves (0.19 mM). The Km of PEPC from green C_4 leaves was reported to be 0.59 mM. Goatly and Smith[60] found that in sugar cane the Kms were 2.9 mM for green leaves and 0.7 mM for etiolated. It could be concluded from these observations that PEPC isolated from green leaves has a higher Km for PEP than that isolated for etiolated leaves.

It is not easy to compare Kms since these depend on the conditions of assay. For example Miziorko et al.[106] found that the Km of PEPC from spinach leaves ranged from 0.1 mM to 1.1 mM depending on the

concentration of manganese ions in the assay medium. It is therefore clear that PEPC assay conditions must be identical if any differentiation between plants based on PEPC activity is required.

The response of the PEPC enzymes from barley, maize and Sedum leaves and the barley pericarp to PEP concentration was studied. Furthermore the effect of G6P on the PEPC activity in barley pericarp was examined in order to establish whether this enzyme most resembled those of C_3, C_4 or CAM plants.

Methods

Crude homogenates of barley leaves and pericarps (var. Julia) maize (var. Golden Bantam) and Sedum spectabile leaves were prepared as described in Chapter 2. The medium consisted of 50 mM-tricine KOH buffer pH 7.5, 0.33 M-sorbitol, 1mM-$MgCl_2$, 1mM-$MnCl_2$ and 10mM-mercaptoethanol. In one experiment the concentration of mercaptoethanol was increased to 100 mM.

I Effect of PEP concentration.

Crude homogenates were supplied with sodium (C^{14}) bicarbonate and varying amounts of PEP. PEPC activity was assayed by the incorporation of radioactive carbon into acid stable products (see Chapter 2). Six assays were carried out. A solution (0.03 mls) of PEP was added to initiate the PEPC reaction. This contained sufficient PEP to bring the final concentration within the range 0.25-2.7 mM. Lineweaver-Burk[94], Hanes[64] and Hill plots[62] were then prepared from the initial rates of reaction.

II Effect of G6P concentration on the Activity of Pericarp PEPC.

Assays were carried out as described in Chapter 2, except that 0.25 ml of pericarp homogenate was used instead of 0.30 ml. The remaining 0.05 ml was made up with buffered medium containing sufficient G6P to bring the final concentration within the range of 0 - 10 mM.

Six different concentrations of G6P were used. The results were expressed graphically with the change in PEPC activity expressed as a percentage of activity in the absence of G6P plotted against increasing G6P concentration.

Results

The rate of C^{14} fixation into acid stable compounds was dependant on the concentration of PEP in all tissues studied. Graphs of reaction rate (V) against substrate concentration((S))were of the hyperbolic type common to enzyme catalysed reactions where the co-operativity of the enzyme is not great.

The Lineweaver-Burk plots[94] i.e. 1/V against 1/(S) were approximately linear for PEPC in crude homogenates of barley and Sedum leaves (Figs. 7.1A and 7.2A respectively) but both the maize leaf enzyme and the pericarp enzyme (Fig. 7.4) gave non-linear plots. A value for Km which is most easily derived from the intercept of the Lineweaver-Burk plot on the 1/(S) axis (intercept = - 1/Km) cannot be derived from non-linear Lineweaver plots. The same data when presented as a Hanes plot ((S)/V against (S)) was linear and the Km could be derived from the intercept on the (S) axis (-Km). The Kms of the various PEPC enzymes are given in Table 7.1. The mean values of the Kms of the PEPCs from the various plants sources was 0.19 using medium containing 10 mM-mercaptoethanol. The PEPC from barley(pericarp and leaf) and from Sedum includes this value within one standard deviation of the estimates of Km, but for maize leaf estimates of Km of PEPC do not fall within the range of one standard deviation.

Also shown in Table 7.1 is the Km of pericarp PEPC assayed in 100mM mercaptoethanol. The Lineweaver-Burk plots (not shown) were practically linear. The Km at this concentration of mercaptoethanol was not significantly different from that for maize leaf.

Figure 7.1 Kinetics of the PEPC of Barley Leaf

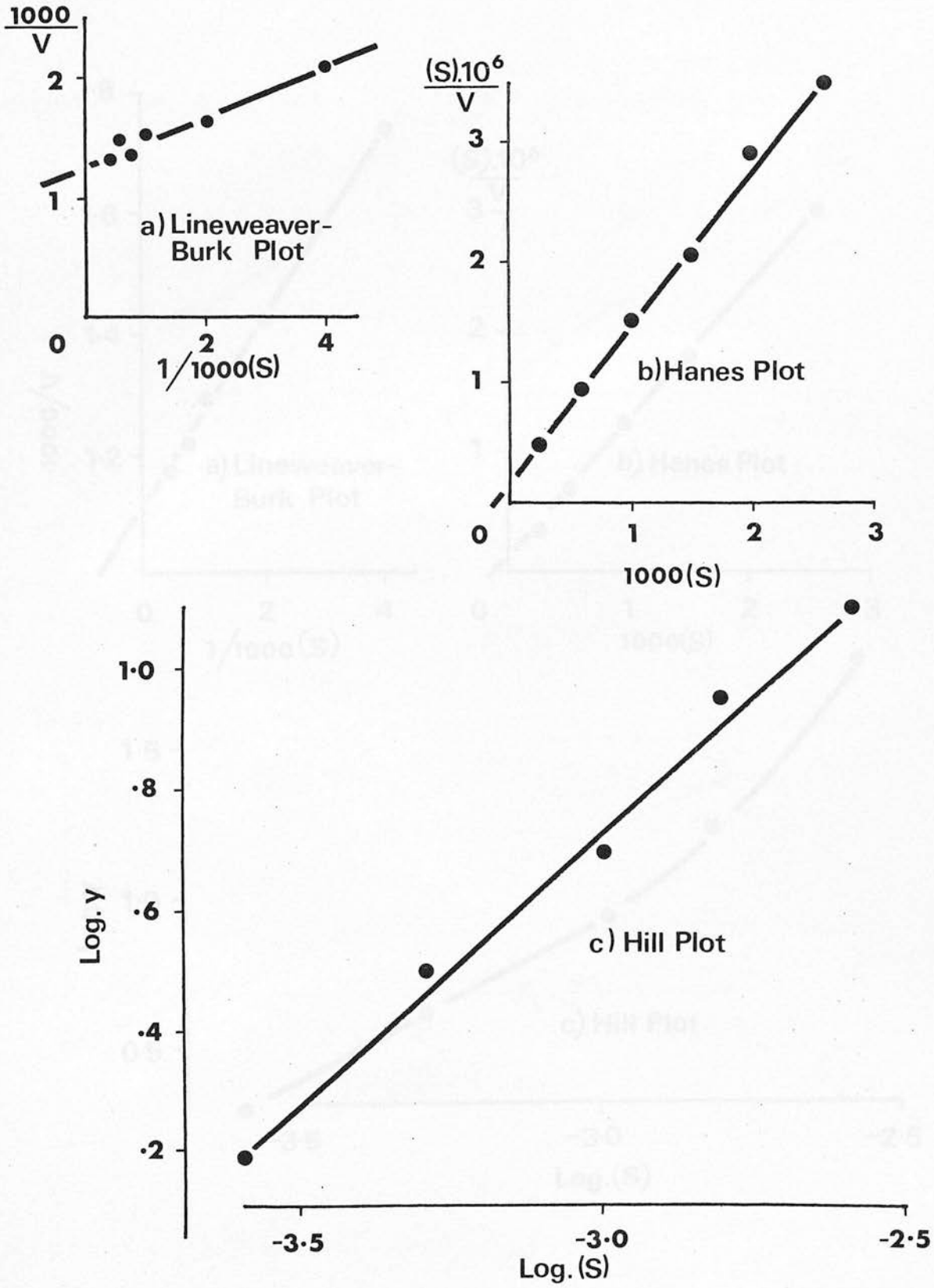


Figure 7.2 Kinetics of the PEPC of a CAM Plant Leaf

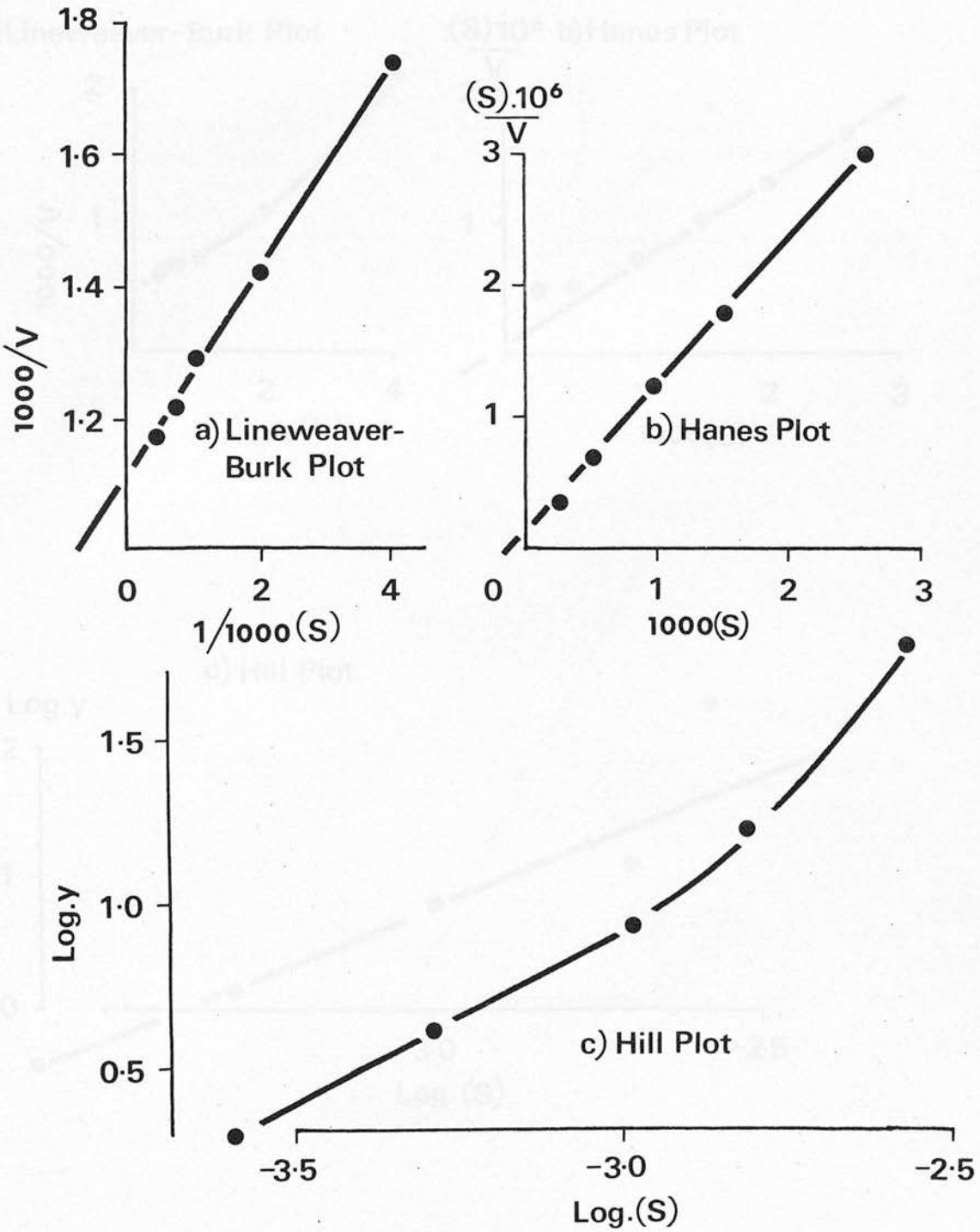


Figure 7.3 Kinetics of the PEPC of Maize Leaf

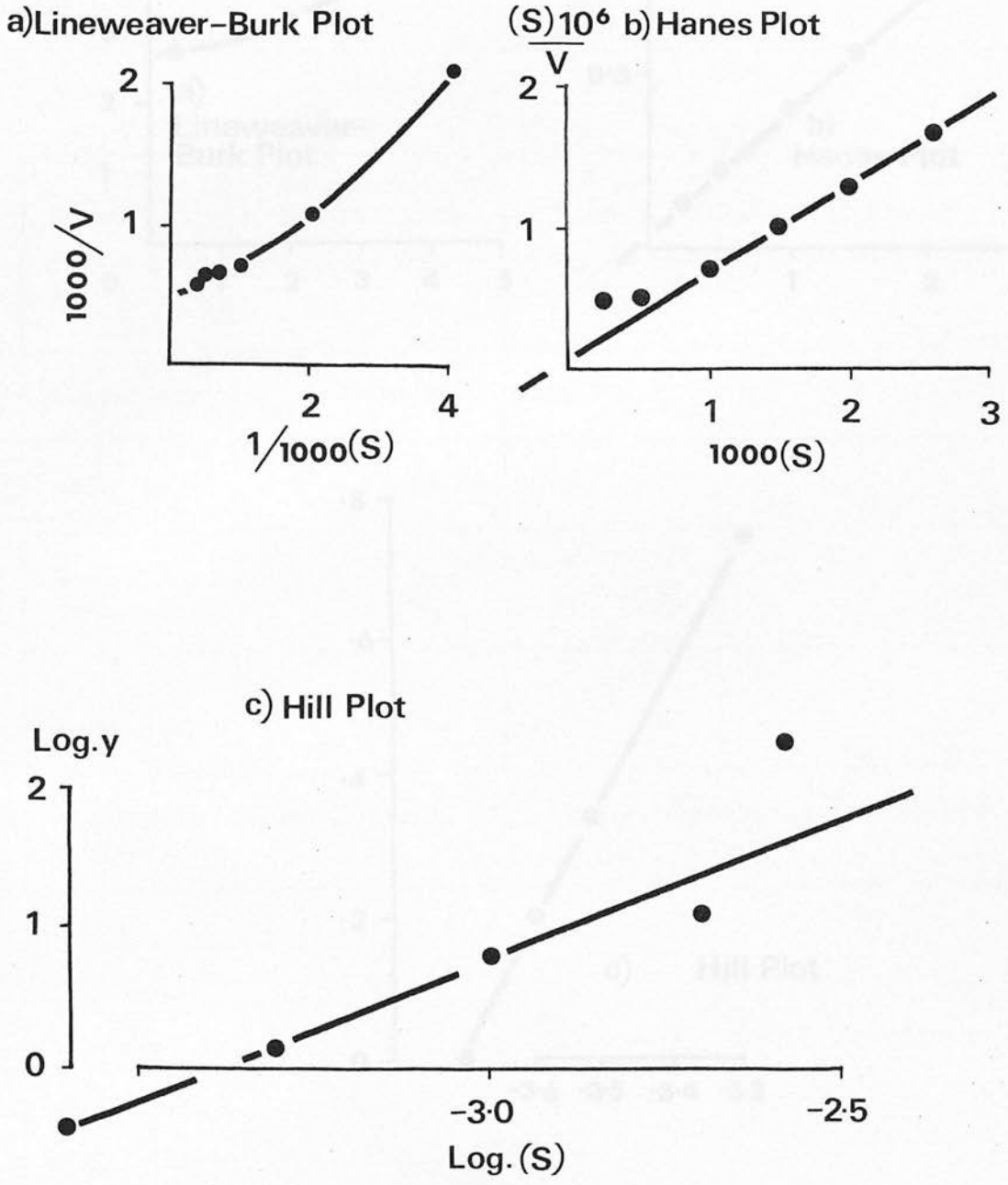


Figure 7.4 Kinetics of PEPC of Barley Pericarp Tissue

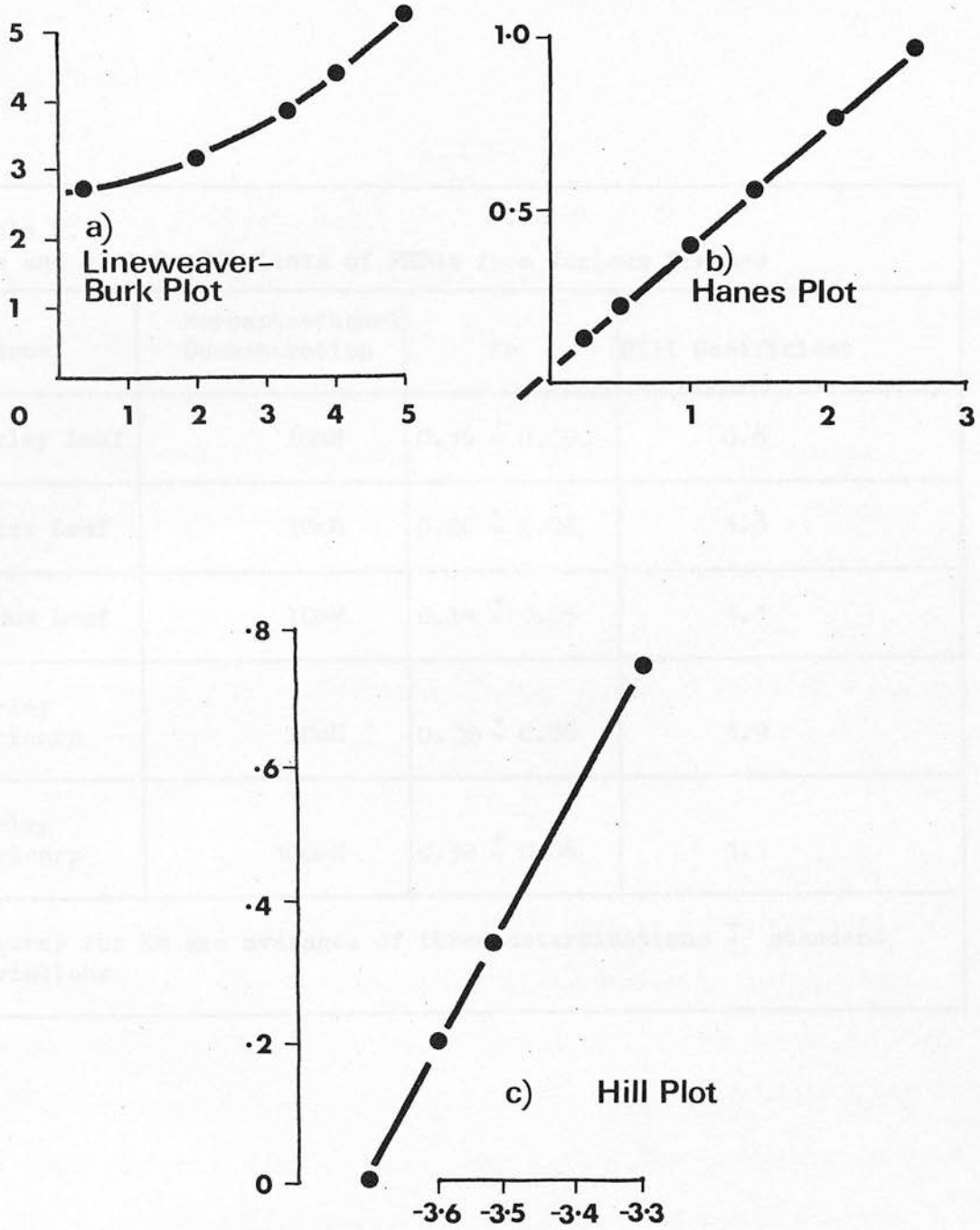


Table 7.1
Kms and Hill Coefficients of PEPCs from Various Tissues

Tissue	Mercaptoethanol Concentration	Km	Hill Coefficient
Barley Leaf	10mM	0.16 ± 0.05	0.8
Maize Leaf	10mM	0.26 ± 0.06	1.8
Sedum Leaf	10mM	0.14 ± 0.05	1.1
Barley Pericarp	10mM	0.20 ± 0.06	1.9
Barley Pericarp	100mM	0.32 ± 0.06	1.1

Figures for Km are averages of three determinations ± standard deviations

While Lineweaver-Burk plots could not always give a value for Km, they were useful in the calculation of the maximum rate of reaction (V max)(intercept on 1/V axis = 1/V max). This quantity was necessary to calculate the approximate fractional saturation of the enzyme (y)

$$y = \frac{V}{V_{max} - V}$$

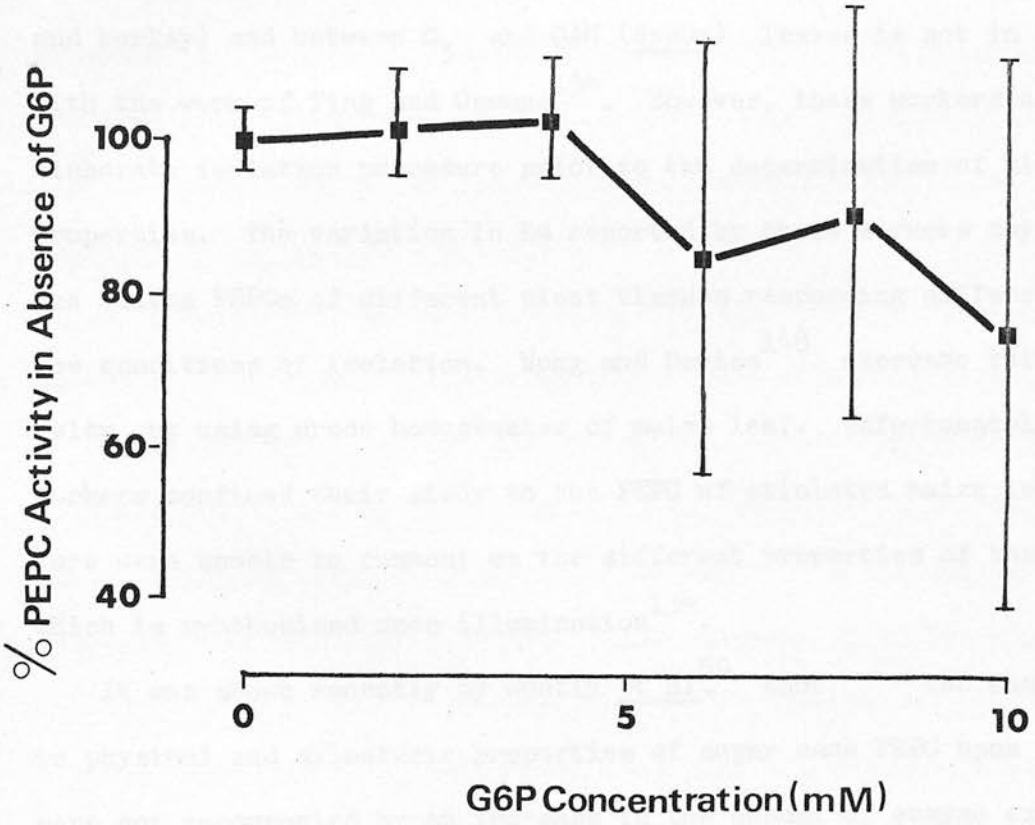
Hill plots (log y against log (s)) are shown in figures 7.1C 7.2C, 7.3C and 7.4C. The Hill coefficients (i.e. the slope of the Hill plots) are given in Table 7.1. The Hill coefficient for barley leaf PEPC is a little less than unity, for Sedum leaf and the pericarp assayed in 100 mM mercaptoethanol it is a little greater than unity. The Hill coefficients of the PEPC of maize leaf and barley pericarp when assayed in 10mM-mercaptoethanol were nearly two.

There was no effect of G6P on PEPC activity (Fig. 7.5). The only effect of increasing G6P concentration appeared to be to decrease the accuracy of the determination.

Discussion

Allosteric enzymes which show a high degree of co-operativity give sigmoidal curves of reaction rate (V) against substrate concentration ((S)). The plots obtained have appeared to be hyperbolic. It is however quite difficult to differentiate between the sigmoid and hyperbolic cruves for cases of intermediate co-operativity in particular where the experimental conditions prevent the determination of V for very small values of (S). Since the transformation of Lineweaver and Burk assumes a hyperbolic function, the plots for allosteric enzymes are rarely linear. Thus the curved Lineweaver-Burk plots of the PEPC in crude homogenates of maize leaf and barley pericarp may be taken as an indication of allosteric properties.

Figure 7.5 Effect of G6P on the Activity of PEPC of Barley Pericarp Tissue



The error involved in measuring small differences in reaction rate which occur at nearly saturating concentrations of substrate lead to a scatter of points near the 1/V axis of the Lineweaver-Burk plots of barley leaf enzyme. This scatter is eliminated to a large extent in the Hanes plot - hence the use of this plot to obtain Km values. The Hanes plot is also not so sensitive to the deviation of allosteric enzymes from the hyperbolic V against (S) curves. Thus, using the same data as was used in the Lineweaver-Burk plots, almost linear Hanes plots were produced for the PEPCs of all tissues. These linear Hanes plots allowed the determination of the Km of PEPC with respect to PEP for each of the tissues studied.

The lack of variation of Km between C_4 and C_3 plant leaves (maize and barley) and between C_3 and CAM (Sedum) leaves is not in agreement with the work of Ting and Osmond[134]. However, these workers used an elaborate isolation procedure prior to the determination of kinetic properties. The variation in Km reported by these workers may have been due to the PEPCs of different plant tissues responding differently to the conditions of isolation. Wong and Davies[148] overcame this difficulty by using crude homogenates of maize leaf. Unfortunately these workers confined their study to the PEPC of etiolated maize leaf and therefore were unable to comment on the different properties of the enzyme which is synthesised upon illumination[134].

It was shown recently by Goatly et al.[59] that the changes found in physical and allosteric properties of sugar cane PEPC upon greening, were not accompanied by an increase in the amount of enzyme extracted from the leaves. The possibility that no new enzyme was synthesised was also indicated by an experiment in which the increase in buoyant density of the enzymes PEPC and acid phosphatase was followed when the leaf was supplied with deuterium oxide during greening. The buoyant

density of acid phosphatase was found to increase due to the incorporation of the deuterium oxide during protein synthesis. There was no such increase in buoyant density of PEPC. Thus, the change in properties of PEPC of etiolated and green leaves may be in response to an effector and not the synthesis of a new species. If this was the case, it is likely that in some conditions the properties of the enzyme of green leaves would revert to those of the etiolated leaf. Hence there would cease to be any significant difference in the properties of the various PEPCs. It would appear therefore that the conditions used here may have caused a shift in the properties of the PEPC of the green C_4 leaves towards that of etiolated C_4 leaves and hence there was little difference in the Km values. There was, however, some evidence of allosteric behaviour in the PEPC of maize leaf and barley pericarp as shown by the non-linear Lineweaver-Burk plots and the Hill coefficients. The Lineweaver-Burk plots for these tissues resemble those of the PEPC of Pennisetum purpureum, a C_4 plant[33].

Allosteric enzymes have a Hill coefficient of greater than unity[62]. In the case of complete co-operativity, where substrate must be bound at every available binding site before the reaction can proceed, the value of the Hill coefficient is equal to the number of binding sites. More usually however the absence of substrate at a binding site merely decreases the catalytic activity of the enzyme. Thus, the Hill coefficient gives the minimum number of binding sites on the protein molecule. The highest values of Hill coefficient were recorded for the PEPCs of tissues which showed non-linear Lineweaver-Burk plots. The results indicate that the PEPC enzymes of maize leaf and barley pericarp have at least two binding sites for PEP. The enzyme from Pennisetum purpureum has been reported to have a Hill coefficient of two. After purification with DEAE cellulose, a Hill coefficient of three

in high concentrations of PEP was reported[33].

The assay of PEPC activity at a higher concentration of mercaptoethanol appears to have destroyed allosteric activity of the enzyme as the Hill coefficient falls almost to unity. This would imply that the subunits of the enzyme are held together with disulphide bonds. The inhibition of allosteric response to PEPC by thiol may explain the differential effect of thiols on the activity of PEPC as compared with their effects on RBPC reported in Chapter 2.

It is possible that the decrease in rate of photosynthesis observed when the pericarp is removed from the endosperm is due to interruption in the supply of an effector of PEPC activity which is produced within the endosperm and subsequently transported to the pericarp. G6P has been reported to be an effector of PEPC[60, 135, 149], yet no such increase in activity was observed in the PEPC from pericarps. This again may have been a response to the conditions of assay.

Conclusion

Evidence based on Lineweaver-Burk plots and measurement of Hill coefficients suggest a strong similarity between the PEPC of green maize leaves and of barley pericarps.

Future Work

The preceding chapters have shown that the pericarps of cereals have some unique features. Their metabolic properties are very similar to those of C_4 plant leaves. Furthermore the rates of photosynthesis as measured by oxygen exchanges indicates that the pericarp may be responsible for nearly one third of the dry matter recovered in the grain at harvest. It might be possible therefore to improve the yield of barley by selectively breeding for pericarps of improved photosynthetic ability. Such a possibility is now discussed both in the light of the results presented here as well as those of other workers.

Intermediate Properties of C_4 like C_3 plants

Menz *et al*.[102] have described a method for screening large numbers of cereal plants for C_4 character, based upon the measurement of compensation point (see Chapter 1). Seedlings of C_3 plants were enclosed in a transparent air tight chamber together with some C_4 plants. As photosynthesis progressed, the concentration of CO_2 in the chamber reached the compensation point of the C_4 plants i.e. the CO_2 concentration was below the compensation points of a classical C_3 plant. In such conditions a classical C_3 plant is unable to fix CO_2 and eventually dies. Hence any plant with a compensation point lower than that generally observed would survive longer than those with a compensation point characteristic of 'normal' C_3 plants. However, these workers have not succeeded in isolating a single variety of small grained cereal plant with a low compensation point.

It is clear, however, from the work described here that there are a number of reasons why the C_4 character of cereal plants has not been previously discovered. Firstly, if a cereal is to outlive classical C_4 plants in a closed chamber, it must have a compensation

point equal to or below that of the C_4 plants. It would not then be possible to identify varieties which had compensation points intermediate between those of classical C_3 and C_4 species. Many characteristics of C_4 plants have been found in plants previously classified as C_3 (Chapter I). However, these do not include the very low compensation point typical of classical C_4 plants. Some properties of the barley pericarp were found to be intermediate between those of classical C_4 and C_3 species e.g. the ratio of chlorophyll a concentration to chlorophyll b concentration (as measured by the method of Wintermans and Demots[147]), the level of activity of PEPC (on a mg chlorophyll basis) and the rate of CO_2 fixation (as measured in attached pericarps).

The simple experimental method of Menz et al.[102] thus requires some modification. This could be done simply by controlling the CO_2 level in the chamber at a specific concentration between the compensation points of C_3 and C_4 plants. Those plants which survived would have (at most) an intermediate compensation point.

Stage of Plant Development

The green pericarp appears only at a specific stage of ear development i.e. at anthesis and has a life of about 35 - 40 days compared with a probable 150 day life span of the whole plant. The C_4 like character may therefore be manifest only at certain stages in the plants development. The use of seedlings in the experiments of Menz et al.[102] therefore has obvious limitations. On the other hand, the closed box technique for detecting low compensation point may not be applicable to more mature plants as the greater internal reservoir of carbohydrate in the larger plant would mean that a longer time period would be required before such plants were deprived. In such a case maturity may be reached before CO_2 depletion had any effect.

The work of Willmer and Johnston[146] showed that the outer layers

of the tomato fruit are capable of fixing CO_2 by the C_4 pathway. This result together with reports of high levels of PEPC in the testa of developing pea[72] and in the pericarps of oats and wheat (shown here and elsewhere[148]), together with the probable existence of the C_4 pathway in the barley pericarp indicates that the seeds and fruit of a number of C_3 plants may exhibit at least some C_4 characteristics. Further work is undoubtedly required in this area.

Function of the C_4 Pathway in C_3 Plants.

Although it is generally agreed that C_4 plants are higher yielding than C_3 plants [151,22], the benefit to be gained by developing a range of crops with C_4 character for temperate climates has been questioned[58]. It was thought probable that C_4 photosynthesis would not be possible in temperate climates[58], particularly as Ehleringer and Björkman[46] have shown that the quantum efficiency of CO_2 fixation at temperatures below 25°C was greater for the classical C_3 plant Encelia californica than for the classical C_4 plant Atriplex rosea. Furthermore Slack et al.[123] have shown that low night temperatues reduce the C_4 properties of maize leaves. However, a far wider range of plants have to be studied before any firm conclusions are reached as to the relative efficiencies of C_4 and C_3 plants in temperate climates. The plant Spartina townsendii which has the specialised anatomy of C_4 plants[95], has a low compensation point even when grown in Britain. The authors [95] claim that this indicates that C_4 photosynthesis is functional in temperate regions, however it remains to be shown that these plants have all the characteristics of classical C_4 photosynthesis. Even if it is established that C_4 photosynthesis can function in temperate climates, it must be determined whether or not this confers any advantage[58].

Fruit and seed maturation usually occurs at the time of the year when there is maximal temperature and light intensity. In these con-

ditions C_4 photosynthesis is more efficient than C_3 photosynthesis[35,46]. The high light intensities and temperatures of summer may then be exploited by the green outer tissues of the immature tomato by C_4 or similar photosynthesis as suggested by Willmer and Johnston[146]. However, this does not appear to be the case for the barley pericarp as it is surrounded by other tissues which may reduce the incident light intensity by as much as 78%[49].

The C_4 pathway may be regarded as a sequence of reactions which provide CO_2 for the Calvin cycle[140]. The allosteric nature of PEPC (Chapter 7) thus provides a mechanism by which the amount of CO_2 supplied to RBPC may be regulated.

The regulation of photosynthesis in the barley pericarp may be complex. It was found (Chapter 5) that the metabolic activities of both the green layer of the pericarp and the endosperm may be controlled by the relative concentrations of oxygen and carbon dioxide in the space enclosed by the transparent layer of the pericarp. In this context, the observation of Huber and Edwards[76] that the rate of $C^{14}O_2$ fixation in C_4 plants increased with increasing oxygen concentration (unlike C_3 photosynthesis which is inhibited by oxygen) is very relevant. Since oxygen is produced by photosynthesis within the pericarp and may remain in contact with this tissue, C_4 photosynthesis would be of greater advantage to the pericarp than C_3 photosynthesis. It has yet to be shown however that the rate of CO_2 fixation by the pericarp tissue is affected by oxygen. Problems would arise if crude tissue homogenates were used in such studies because of the presence of polyphenol oxidase and phenolic substrates (see Chapter 2).

Differences between C_3 and C_4 plants

The recent observation of Goatley et al[59] (see chapter 7) that the

PEPC of etiolated sugar cane leaves changes from its C_3 form (low activity not affected by G6P) when the leaves green on introduction to the light without associated protein synthesis indicates that the enzymes activity is modified by metabolites in the greening leaf. This may implicate that any leaf PEPC may be modified by metabolites to produce C_4 like enzyme. There may be no inherent biochemical difference between C_3 and C_4 plants, rather that in C_4 plants a greater proportion of the PEPC is in what might be termed the C_4 state.

The present work suggests strongly that the PEPC of the barley pericarp does indeed adopt C_4 properties (Chapter 7) in an otherwise typically C_3 plant. Similarly, it is likely that the PEPC in the skin of the unripe tomato undergoes a change in form from the C_3 to the C_4 type.

Goatley et al.[59] hypothesised that changes in the properties of the PEPC of etiolated sugar cane leaves when placed in the light were due to the polymerisation of the enzyme. There are recent reports[48] that the PEPC of the C_4 plant Tidestromica oblongifolica native to arid environments, has a molecular weight twice that of the PEPC from Atriplex sabulosa (also a C_4 plant) which is found in oceanic habitats. The distinction between C_3 and C_4 plants on the basis of the allosteric properties of PEPC is thus arbitary and probably unreliable. Instead there may be a gradation of properties from classical C_3 through C_4 like C_3 plants C_4 to 'extreme' C_4 plants each adapted to its own particular environment. If this is indeed the case, it could be argued that there would be little benefit in developing C_4 character in range of C_3 crops. However, in barley and tomato it appears that the ability to develop C_4 character is limited to the fruit and the range of C_4 character displayed by these tissues is probably dependent

on the degree of polymerisation of PEPC.

The extent of C_4 character which a plant may exhibit is obviously limited by its genetic makeup. Thus, the breeding of C_4 like C_3 plants may result in a greater utilisation of the high temperatures and light intensities of summer in a greater number of tissues. This ability would also make them suitable crops for cultivation of latitudes nearer the equator than the original species, thus increasing the land area available for cultivation. Such plants however may show the reduced photosynthetic efficiency of C_4 plants in temperate conditions.

The first stage of a breeding programme to develop a C_4 like cereal plant would be the assay of C_4 character over a wide range of cereals. This initial survey may well indicate varieties with much more C_4 character than barley var. Julia. The breeding programme itself would greatly increase our knowledge of photosynthetic CO_2 fixation since it should be possible by studying a range of plants with intermediate photosynthetic properties to ascertain which metabolic and genetic processes are involved in controlling the expression of C_4 character.

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EVIDENCE FOR C_4 PHOTOSYNTHESIS IN BARLEY PERICARP TISSUE

Anthony R. Nutbeam and Carol M. Duffus

Department of Agricultural Biochemistry
School of Agriculture, University of Edinburgh,
West Mains Road, Edinburgh EH9 3JG, Scotland.

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SUMMARY: The products of photosynthetic carbon dioxide fixation were determined in isolated pericarps of immature barley grains. Of the carbon dioxide fixed after 1 min photosynthesis 84% was in the C_4 acid malic acid. The remaining label was in hexose phosphates and sucrose. By 2 min sucrose was the major labelled product and at 6 min accounted for 94% of the total carbon dioxide fixed.

C_3* and C_4 plants are associated with certain physiological and anatomical characteristics. For example, in comparison with C_3 plants, C_4 plants have a low carbon dioxide compensation point (1) and high rates of photosynthesis (2). In addition they have a specialised type of leaf anatomy (3) in which the central bundle sheath cells are surrounded by outer or mesophyll cells. The leaves of C_3 plants have essentially a single type of cell specialising in photosynthesis. Thus, the temperate plants barley and wheat have been classified as C_3, and the tropical plants maize and sugar cane as C_4.

It is noteworthy, however, that this division of plants into groups is based entirely on physiological and anatomical properties of the leaves. It is well established, however, that the dry matter entering the grain of cereals is derived from photosynthesis after ear emergence (4) and that it is the ear itself, together with the flag leaf, which makes the major contribution to grain carbohydrate (5,6). Indeed at the time of ear emergence many of the lower leaves are senescent.

*Abbreviations: C_3 plants, plants which have only the reductive pentose phosphate pathway in photosynthesis; C_4 plants, plants which have both C_4 dicarboxylic acid pathway and reductive pentose phosphate pathway in photosynthesis; CAM plants, succulent plants with Crassulacean acid metabolism.

The tissues of the ear which contain chlorophyll and which may contribute directly to grain carbohydrate include the awns, glumes, paleae and pericarp. The awns are certainly capable of photosynthesis and of supplying carbohydrate to the grain (7) although under certain conditions awned plants yield less than non awned plants of the same variety (8). The glumes and paleae are dull green, loosely adhering protective tissues which surround the grain. They later become part of the husk of the mature grain. Nothing is known of their ability to fix and transport carbon dioxide into the immature grain. The pericarp of immature cereal grains is a bright emerald green tissue surrounding the outer cells or aleurone layer of the endosperm. It is contained in a transparent "bag" of tissue probably only one or two cells thick. Thus, the pericarp is distinct in structure and location from the leaves. If, additionally, it has different biochemical characteristics the situation could arise where the plant is C_3 with respect to the leaves and C_4 (for example) with respect to the pericarp. Previous work (9) has reported the presence, in the barley pericarp, of levels of phosphoenol pyruvate carboxylase (PEPC; EC 4.1.1.31) higher than those usually found in the leaves of C_3 plants. This enzyme catalyses the primary carboxylation reaction in C_4 plants. Phosphoenol pyruvate synthetase, an enzyme of the C_4 dicarboxylic and pathway, was also reported to be present.

This work describes the metabolic events following carbon dioxide fixation by isolated pericarps. Results with leaves of maize (C_4), pea (C_3) and *Sedum spectabile* (CAM) are included for comparison.

MATERIALS AND METHODS

Plants were grown in greenhouses with natural daylength extended to 18 h with mercury vapour lamps. Barley grown in these conditions produced grain which reached maturity 60 days after anthesis. Ears were removed from the growing plants 25-30 days after anthesis, immediately prior to isolation of the pericarps. At this stage in development the grain was still increasing in size, both layers of the pericarp were fleshy and the chlorophyll content

of the green layer was at its maximum value (9). The paleae, lemma and transparent layer of the pericarp were peeled from the grain. The green layer was then carefully removed and quickly transferred to glass fibre discs and incubated with $^{14}CO_2$ using a technique similar to that described by Coombs and Baldry (14). After incubation the pericarps were washed and then homogenised in ethanol at 70°C. The alcohol extract was centrifuged at 1,000 g for 2 min, and 5 µl of the supernatant counted for radioactivity. The residue was re-extracted in water for 24 h and the total carbon dioxide fixed calculated from the sum of alcohol soluble and water soluble radioactivity. Chlorophyll was determined in the alcohol extract as described by Wintermans and De Mots (15), and the products of carbon dioxide fixation separated electrophoretically as described by Farineau (16).

RESULTS AND DISCUSSION

The rate of photosynthesis by pericarps is low compared to maize, pea and *Sedum* leaves (Fig.1). The low rates may in part be due to the difficulties encountered in preparing the tissue for such experiments. The chlorophyll containing layers of the pericarp are delicate and care must be taken to ensure a minimum of cell damage. Broken cells are then susceptible to further attack by the products of phenol oxidase activity. While the number of pericarps used was the minimum required to achieve sufficient incorporation to identify products, the time taken for their removal from the grain and transfer to glass fibre discs was much longer than that taken for leaf experiments. *In vivo* rates, therefore, are probably higher than those reported here.

In order to obtain measurable rates of photosynthesis with pericarps the partial pressure of carbon dioxide used was 0.1%. Under these conditions pea has a rate of photosynthesis similar to that of maize. Under physiological conditions (0.03% carbon dioxide) the rate of pea photosynthesis would be relatively much less.

High concentrations of carbon dioxide favour the formation of C_3 photo-

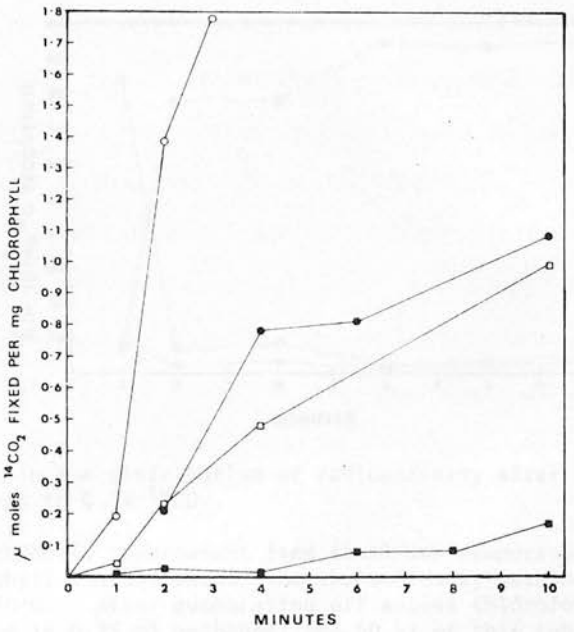

Fig.1. Rate of carbon dioxide fixation by the leaves of *Sedum spectabile*, maize (*Zea mays* var. Golden Bantam), pea (*Pisum sativum* var. Meteor) and pericarps of barley (*Hordeum distichem* var. Julia).

Pericarps (10 per disc) were placed on glass fibre discs (21 mm diameter) soaked in isotonic buffer (50 mM tricine KOH buffer, pH 7.5; 0.33 M sorbitol) to prevent dehydration. Three such discs were placed in a Perspex chamber of 11 ml volume and 20 μl of a 17 mM solution of sodium (^{14}C) bicarbonate (specific activity approximately 60 Ci/Mole) were injected through a rubber seal into a well containing 0.15 ml lactic acid, such that the concentration of carbon dioxide in the chamber was 0.1%. The chamber was illuminated for periods of time from 1 minute to 10 minutes with a tungsten halogen lamp (incident light intensity 17,000 Lux) at 25°C.

O–O *Sedum spectabile*; ●–● *Pisum sativum*; □–□ *Zea mays*; ■–■ *Hordeum distichum* pericarp.

synthetic products (10). It was therefore surprising that, of the carbon dioxide fixed in barley pericarps after 1 min photosynthesis, 84% was in the C_4 acid malic acid (Fig.2). The remaining label was in hexose phosphates and sucrose. By 2 min sucrose was the major labelled product and at 6 min accounted for 94% of the total ^{14}C fixed. Similar results have been obtained for wheat pericarp. At the same time normal C_3 photosynthesis was observed with pea (C_3) leaves. These results for cereal pericarp closely resemble

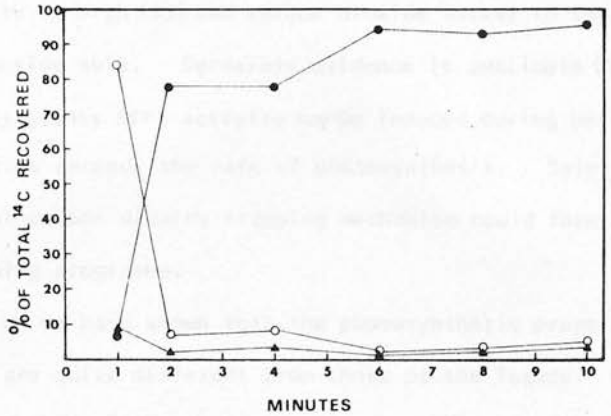

Fig.2. Changes in the distribution of radioactivity after exposure of pericarps to 0.1% $^{14}CO_2$.

The alchoholic supernatant (see text) was evaporated to dryness. Chlorophyll was extracted from the solids by washing twice with 0.5 ml chloroform. After evaporating off excess chloroform the solids were taken up in 0.25 ml methanol, and 50 µl of this subjected to high voltage electrophoretic separation (see text). Compounds containing ^{14}C were identified by exposure to photographic film placed in contact with the electrophoretogram for 4 weeks. Areas containing ^{14}C were punched from the paper and the radioactivity determined by liquid scintillation counting (efficiency 80%).

●–● sucrose; O–O malate; ▲-▲ hexose phosphate.

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those obtained by Hatch (11) for photosynthesising maize and sugar cane leaves in which malate was also the initial product of carbon dioxide fixation. Thus, while the barley pericarp may not have the anatomical and physiological properties thought to be characteristic of C_4 plants it is capable of C_4 photosynthesis.

The role played by the pericarp in the deposition of grain carbohydrate is not clear. In particular the origin of the carbon dioxide fixed is unknown. The transparent layer of tissue surrounding the green layer of pericarp does not appear to have stomata. Thus, it may be impermeable to atmospheric carbon dioxide. It is possible, therefore, that the function of the pericarp is to prevent respiratory losses from the endosperm by refixing respired carbon dioxide. A similar function has been suggested for the PEPC of the testa of developing peas (12). At this period in endosperm development

metabolic activity is high (13) and carbon dioxide losses in the absence of PEPC could be considerable. Certainly evidence is available (12) to support the theory that in C_3 plants PEPC activity may be induced during periods when the rate of respiration exceeds the rate of photosynthesis. Selection of plants with an efficient carbon dioxide trapping mechanism could form the basis of a successful breeding programme.

In conclusion we have shown that the photosynthetic properties of the barley pericarp are quite different from those of the leaves. The first formed product of photosynthesis is the C_4 acid malate which is then rapidly converted to sucrose. It seems likely that this is a result of the unique environment of the pericarp, situated as it is, between an impermeable transparent layer and the rapidly respiring endosperm. Thus, barley embodies some of the features characteristic of C_4 or tropical plants.

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Work on oxygen exchange in detached grains of barley and wheat (described in Chapter 4) has been accepted for publication by Plant Physiology. Reprints are not yet available.